Molecular typing of *Toxoplasma gondii* isolates from cats and humans in Germany

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Abstract

Toxoplasma gondii is a protozoan parasite that can infect almost all warm-blooded animals, including birds and humans. The definitive host is the cat which excretes the highly infectious and environmental resistant oocyst stage. Unlike other coccidian parasites, such as *Neospora caninum* and *Eimeria*, *T. gondii* does not wholly rely on its sexual stage in felids to successfully be transmitted to other hosts and host species. Equally important for the spread of *T. gondii* is the transmission of *T. gondii* by ingestion of contaminated food (prey) between host species. In fact, sexual recombination is a relatively rare event observed in *T. gondii*. This is one of the reasons why a clonal population structure of *T. gondii* is observed. In Europe and North America the majority of genotypes (types) of *T. gondii* are members of only three canonical clonal types, designated type I, type II and type III. In South America and Asia, *T. gondii* is shown to have an increased genetic diversity with a high prevalence of non-canonical or atypical genotypes. However, even within those non-canonical or atypical genotypes, a clonal population structure can be observed. More importantly, mouse virulence of types I, II and III differ markedly. While infection with *T. gondii* type I is always lethal in mice (Lethal $Dose_{100}$ = 1 parasite), infections with 10^4–10^6 parasites of type II or type III are needed to have the same effect in mice (LD_{50} = 10^3–10^5 parasites). This study shows that the majority of cats in Germany excrete *T. gondii* oocysts of type II. We have not observed any type I *T. gondii*, but show that type III and, importantly, mixed type infection as well as non-canonical *T. gondii* are present in Germany. For the first time we demonstrate that a sexual cross between *T. gondii* type II and type III in a single, naturally infected cat occurred in Germany resulting in excretion of many genetically different non-canonical *T. gondii*. Most of the identified non-canonical *T. gondii* show a high virulence in mice. The RFLP-typing analysis of a limited number of *T. gondii*-DNA isolated from human samples revealed only alleles of *T. gondii* type II. I show that genetic recombination of different *T. gondii* types in Germany can lead to a higher genetic diversity and generation of highly mouse-virulent *T. gondii*.

Zusammenfassung

Toxoplasma gondii weist eine weltweite Verbreitung auf und kann fast alle Wirbeltiere, vor allem Vögel und Menschen infizieren. Felide sind Endwirte von *T. gondii*, welche das infektiöse und umweltresistente Oozysten-Stadium ausscheiden können. Im Gegensatz zu anderen parasitären Erregern, wie zum Beispiel *Neospora caninum* und *Eimeria*, kann *T. gondii* auch ohne sexuelle Vermehrung unterschiedlichste Zwischenwirte und Zwischenwirtspezies infizieren. Obwohl eine sexuelle Phase ein Teil des Lebenszyklus ist, werden rekombinierte Genotypen nur sehr selten beobachtet. Dies ist ein Grund dafür, warum *T. gondii* eine klonale Populationsstruktur erkennen lässt. Während in Nordamerika und Europa drei klonale Genotypen (Typ I, II und III) dominieren, werden in Südamerika und Asien, neben den drei bekannten auch atypische und andere Genotypen beobachtet. Aber auch innerhalb der atypischen Genotypen lässt sich eine klonale Populationsstruktur erkennen. Die klonalen Linien I, II und III weisen Unterschiede in ihrer Virulenz für Mäuse auf. Typ-I-Stämme sind hochvirulent. Die Infektion mit nur einem Organismus führt bereits zum Tod. Klonale Typ-II- und Typ-III-Stämme sind avirulent für Mäuse. Nur Infektionen mit mehr als 10^3 Organismen führen zum Tod. In dieser Studie zeige ich, dass die Mehrzahl isolierter *T. gondii*-Oozysten dem Typ II zuzuordnen ist. Es wurde keine Typ-I-, dafür aber eine Typ-III-Infektion und vereinzelte Hinweise auf Mischinfektionen und nicht-kanonische *T. gondii* Genotypen beobachtet. Erstmalig kann gezeigt werden, dass aus einer Rekombination zwischen den klonalen Typen II und III genetisch unterschiedliche *T. gondii* in einer natürlich infizierten Katze entstanden sind. Die identifizierten nicht-kanonischen *T. gondii* Klone weisen unterschiedliche, meist hohe Virulenz im Mausmodell auf. Eine geringe Anzahl von *T. gondii*-DNA Proben von humanen Toxoplasmose-Fällen deutet auf eine Infektion mit *T. gondii* des Typs II hin. Ich zeige mit dieser Studie, dass sexuelle Rekombination von *T. gondii* in Deutschland möglich ist, und diese zur Entstehung von hochvirulenten *T. gondii* führen kann.

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List of Abbreviations

A	adenine
APC	antigen presenting cell
C	cysteine
Chr.	chromosome(s)
CI	confidence interval
CT	congenital toxoplasmosis
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
d.p.i.	days post infection
EST	expressed sequence tag
FCS	foetal calf serum
G	guanine
GKO	interferon-γ knockout
h	hour(s)
i.p.	intra peritoneal
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulated factor
IRG	immunity related GTPase

JAK	Janus kinase
LD	lethal dose
MHC	Major Histocompatibility Complex
min	minute(s)
mM	mini molar
NFkB	nuclear transcription factor kappa B
NK	natural killer cell
OT	ocular toxoplasmosis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PBS-TG	phosphate buffered saline+tween+gelatine
PCR	polymerase chain reaction
PVM	parasitophorous vacuole membrane
ROP	rhoptry secreted protein
RE	restriction enzyme
RFLP	restriction length polymorphism
RNA	ribonucleic acid
rpm	rotations per minute
SNP	single nucleotide polymorphism
s	second(s)
siRNA	small interference RNA
STAT	signal transducer and activator of transcription
t	time

TLR	toll-like receptor
TNF	tumour necrosis factor
T	thymine
UPRE	unfolded protein response element
x g	centrifugal force

1 Introduction

1.1 General information

Toxoplasma gondii is one of the most prevalent and most successful parasites worldwide. It is thought to be able to infect all warm-blooded animals found in most regions of the world. There is evidence of its existence on almost all continents, Europe, North and South America, Asia, Africa, the Arctic and Australia, except for Antarctica. It can cause a wide spectrum of disease manifestations in its host animals. This ranges from no or malign symptoms to death, such as in sea mammals. It is of major concern in many species, especially in livestock animals as well as humans, with a congenital infection of *T. gondii*. This can lead to damage in the developing foetus or spontaneous abortion.

Initially, *T. gondii* was found in *Ctenodactylus gundi*, a desert rodent in Tunisia that was used for *Leishmania* research at the time, and therefore wrongly believed to be *Leishmania*. However, morphological examination and comparison revealed the discovery of a new parasite by [Nicolle and Manceaux, 1908]. At the same time, [Splendore, 1908] also isolated the same organism from rabbits in Brazil. Years later, *T. gondii* was isolated from different animals but also humans [Sabin, 1939; Wolf *et al.*, 1939].

1.2 Lifecycle of *T. gondii*

One of the major advantages of this parasite is the ability to infect and to persist in almost all warm-blooded animals worldwide. Its lifecycle was first described in 1965 [Hutchison, 1965] but was not fully elucidated until 1970 [Frenkel *et al.*, 1970; Hutchison *et al.*, 1970; Hutchison *et al.*, 1971]**.** An overview of the lifecycle is shown in **Figure 1**.

Toxoplasma life stages can be divided into four major groups: oocysts, tachyzoites, bradyzoites and the sexual stages (micro- and macrogametes).

1.2.1 Oocysts

Unsporulated oocysts are excreted by felids, the definitive hosts of *T. gondii*, within 3-5 days after feeding them tissue cysts [Dubey *et al.*, 1970a]. Oocysts are subspherical to ellipsoidal in shape and measure between 10-12 µm [Dubey, 1998a]. Within 2-7 days they sporulate and only then they are infective for animals and humans. During sporulation two sporocysts are formed inside the oocysts wall, each containing 4 sporozoites. Oocysts are highly environmentally resistant. Desiccation can kill oocysts. While room temperature promotes sporulation, temperatures below 4°C may slow the sporulation process but are not always sufficient to prevent sporulation [Dubey *et al.*, 1970b; Frenkel *et al.*, 1970; Lindsay *et al.*, 2002]. The oocyst stage can also survive UV-treatment and remains infective in water for up to 54 months at 4°C and in soil for 18 months at various temperatures [Dubey, 1998a; Dumetre and Darde, 2003; Frenkel *et al.*, 1975]. Therefore, once contaminated with oocysts, water, soil and vegetation present a major source of *T. gondii* infection to animals and humans. Indeed, major outbreaks of toxoplasmosis in Canada and Brazil could be traced back to contaminated water sources [Bowie *et al.*, 1997; de Moura *et al.*, 2006; Stagno *et al.*, 1980]. Upon ingestion of oocysts by an intermediate host, the oocyst wall is digested in the small intestine of the intermediate host releasing the sporocysts and sporozoites. Sporozoites penetrate into intestinal cells, transform into tachyzoites which start to multiply asexually in the lamina propria from where they may spread to other tissues to transform later on into bradyzoites which finally form tissue cysts [Dubey and Frenkel, 1972; Dubey, 1998a, 1998b; Dubey *et al.*, 1998; Dubey, 2005].

1.2.2 Tachyzoites

Tachyzoites are crescent-shaped and about 6 µm in length. They enter the host cell by actively penetrating the cell membrane. Thereafter, the tachyzoite is surrounded by a parasitophorous membrane (PVM) which consists of both, host- and parasite-derived proteins [Mordue *et al.*, 1999]. Tachyzoites multiply intracellularly by endodyogeny until their growth can no longer be sustained by the host cell. The infected cell then ruptures releasing the tachyzoites. The released parasite stages may then continue to infect neighbouring uninfected cells or can spontaneously convert into the slowly replicating bradyzoite stage within infected cells. The rate of growth was discovered to be strain- and host cell type-dependent [Appleford and Smith, 1997]. Mouse virulent strains of *T. gondii* were shown to grow faster *in-vitro* than avirulent strains. Although *T. gondii* isolates have been genetically classified into types I, II and III, no structural differences among them were apparent [Howe and Sibley, 1995]. Importantly, tachyzoites can be passed from maternal blood to the foetal tissue causing congenital *T. gondii* infections.

1.2.3 Bradyzoites

Bradyzoites, which form tissue cysts, can be found as early as three days post inoculation (d.p.i) in mice [Dubey and Frenkel, 1976]. The process of stage conversion from fast-dividing tachyzoite to slow-dividing bradyzoites seems to be a spontaneous process occurring when the replication rate of tachyzoites slows down. Consistent with this observation, rapidly dividing *T. gondii* strains, such as RH (type I), form bradyzoites less readily [Bohne *et al.*, 1994; Weiss and Kim, 2000] than other strains. Stage conversion can be induced *in-vitro* by temperature stress and pH-stress [Soete *et al.*, 1994; Weiss *et al.*, 1995]. Bradyzoites can also form when a host ingests oocysts, to a small extent tachyzoites or contaminated meat with tissue cysts. It is believed that tissue cysts can periodically rupture thus releasing parasites that re-invade host cells and establish new tissue cysts [Reiter-Owona *et al.*, 2000].

1.2.4 Sexual stages

Sexual stages can only develop in felids, the definitive host of *T. gondii*. Felids can get infected with *T. gondii* by ingestion of sporulated oocysts or by ingesting tissues of infected intermediate hosts, e.g. rodents or birds. In both cases, the cell wall surrounding bradyzoites, oocysts or the sporocyst will be digested by enzymes and the acidic environment of the stomach and enzymes of the intestine, to release bradyzoites or sporozoites. These stages undergo several rounds of asexual propagation in epithelial enterocytes followed by the formation of sexual stages (gametocytosis). A few microgametocytes (males) containing 20-30 microgametes, and large numbers of macrogametocytes (females) are formed. Mature macrogametes contain all nutrients required for oocyst formation [Ferguson *et al.*, 2005]. During sexual propagation, microgametes will invade a single macrogamete thus fertilising it. This process leads to the formation of a diploid zygote that will later develop into the oocyst stage. Interestingly, only macrogametogony is associated with the synthesis of wall-forming bodies which are required to form the oocyst wall [Ferguson, 2002].

When oocysts are released with feline faeces, they undergo meiosis in the environment generating four haploid sporozoites. The prepatent period for oocyst shedding in cats is 3–10 d.p.i. after bradyzoite take-up and 18 days or more after ingesting oocysts. The prepatent period is reported to be strain-independent [Dubey, 2005]. Millions of oocysts can be shed over a period of 14 days by a single cat. After sporulation of oocysts in the environment, for example in water, on vegetables, food stuff, cat litter, in soil etc., they may be taken up by a wide range of intermediate hosts, such as wild and livestock animals, birds or humans. In the intermediate host, tachyzoites are formed first, followed by the formation of tissue cysts. Tissue cysts are mainly found in the central nervous system (CNS), the eye and striated as well as unstriated muscular tissue. However, bradyzoites were also shown to be present in visceral organs such as the lungs, liver and kidneys [Dubey, 1998c]. Tissue cysts are considered the terminal life-stage in the interme-

diate host. They may persist life-long. Importantly, tissue cysts are also infectious for other host species [Dubey, 1998d].

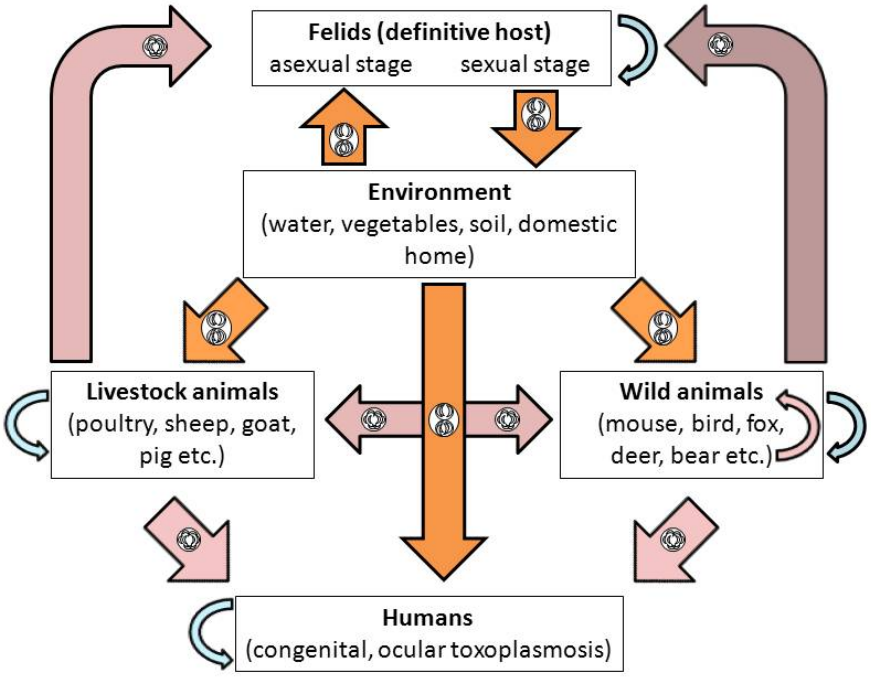

Figure 1: Life cycle of *T. gondii*.

Orange arrow: transmission by oocysts, pink arrow: transmission by tachyzoites and/or bradyzoites, curved pink arrow: transmission between species in the wild (prey-predator relationship), curved blue arrow: vertical transmission (mother to offspring).

What distinguishes *T. gondii* from other cyst-forming coccidian is the fact that horizontal transmission is not restricted to oocysts, i.e. from definitive to intermediate hosts, but also via horizontal transmission by tissue cysts, i.e. between intermediate hosts. Another route of infection was shown to be initiated by tachyzoites which can be passed vertically from the mother to the offspring. This has been demonstrated to be true for the definitive (felids) and several intermedi-

ate host species such as mice [Aramini *et al.*, 1998], rats [Dubey, 1997], sheep [Innes, 2009] but also humans [Dunn *et al.*, 1999; Minkoff *et al.*, 1997]. All three life-stages (oocysts, tachyzoites, and bradyzoites) are infectious to their host species. In the context of parasite survival, it follows that *T. gondii* may be transmitted from definitive to intermediate hosts, from intermediate to definitive host as well as between definitive and between intermediate hosts. In settings where domestic felids are absent, wild felids such as jaguars, lynx or bobcats can serve as definitive hosts [Demar *et al.*, 2008; Garcia-Bocanegra *et al.*, 2010; Millan *et al.*, 2009a; Mucker *et al.*, 2006]. Furthermore, even in the absence of a definitive host, *T. gondii* may persist in the environment by cycling only between intermediate host species. Due to this efficient life cycle that includes not only domestic but also wild animals, it has been suggested that the *T. gondii* life cycle may consist of two elements, the domestic and the sylvatic (wild) cycle. Although the two cycles may exist separately, for example in case of geographic boundaries, exchange will be possible at the edge where spill-over may occur [Grigg and Sundar, 2009]. At these edges, may it be boundaries of the rainforest and anthropised coastal regions of Guiana or the boundary between forest and sub-urban regions in Germany, transmission between the sylvatic and the domestic cycle is highly likely [Mercier *et al.*, 2011].

1.3 *T. gondii* prevalence in cats and humans in Germany and Europe

Due to its wide host-range and efficient life cycle, *T. gondii* is highly prevalent in animals and humans worldwide. *T. gondii* oocyst shedding in cats is difficult to assess due to the short patent period. Therefore, only few studies were undertaken to analyse oocyst shedding. Most studies consisted of microscopic faecal examinations and in some cases these findings were confirmed by a *T. gondii*-specific PCR. This fact is important since *T. gondii* cannot be readily distinguished from related oocysts forming protozoan parasites, such as *Hammondia*. Despite the development of molecular methods enabling the distinction of oocysts of these species, not many studies applied such methods [Schares *et al.*, 2008b;

Sreekumar *et al.*, 2005]. Therefore, reports on oocysts need to be studied more closely. Comparison of such publications is difficult since different approaches of sampling and different methods of detection were applied. Some studies used all the faecal matter available [Schares *et al.*, 2008b] while others examined only a standardised amount [Miro *et al.*, 2004].

Table 1 gives an overview of feline faecal samples of domestic cats examined for the presence of *T. gondii* oocysts in Germany and Europe. Seroprevalence data suggest that the prevalence of domestic cats infected with *T. gondii* ranges between 26.00 and 65.00% (**Table 2**). However, the seroprevalence in wild cats (*Felis sylvestris*) was found to be higher with 50–100% (**Table 3**). [Miro *et al.*, 2004] reported that *T. gondii* seroprevalence in stray cats was statistically significantly higher than in domestic or farm cats. Several studies also reported a statistically significant correlation between the presence of high antibody titres and higher seroprevalences in older cats (older than one year) compared to low antibody titres and low seroprevalences in younger cats [De Craeye *et al.*, 2008; Miro *et al.*, 2004]. Therefore, primary infection with *T. gondii* will often occur in cats aged one year or younger and re-infection with *T. gondii* may therefore more frequently occur later in life.

Table 1: Coprological examinations of domestic cats for the presence of *T. gondii* oocysts in Germany and Europe.

AUT: Austria; CHE: Switzerland; CZE: Czech Republic; ESP: Spain; FRA: France
M: microscopic examination; B: bioassay in mice

Country	No. of samples analysed	Method	No. of positive samples (%)	Reference
GER	24106	M, PCR	26 (0.11)	[Schares *et al.*, 2008b]
GER	3167	M	142 (4.50)	[Barutzki and Schaper, 2003]
GER	8560	M	66 (0.8)	[Barutzki and Schaper, 2011]
AUT	1368	M	27 (2.00)	[Edelhofer and Aspöck, 1996]
CHE	252	M, PCR	1 (0.40)	[Berger-Schoch *et al.*, 2011b]
CZE	390	M, B, PCR	0 (0.00)	[Svobodova *et al.*, 1998]
ESP	382	M	0 (0.00)	[Miro *et al.*, 2004]
ESP	592	M	0 (0.00)	[Montoya *et al.*, 2008])
FRA	322	M	0 (0.00)	[Afonso *et al.*, 2006]

Table 2: Serological examinations of domestic cats for *T. gondii* antibodies in Germany and Europe.

AUT: Austria; BEL: Belgium; CZE: Czech Republic; ESP: Spain; FRA: France; HUN: Hungary

Country	No. samples analysed	Method	No. of positive samples (%)	Reference
GER	306	ELISA	138 (45.09)	[Tenter *et al.*, 1994]
GER	300	ELISA	197 (65.60)	[Hecking-Veltman *et al.*, 2001]
AUT	456	IFAT	220 (48.24)	[Edelhofer and Aspöck, 1996]
BEL	567	IFAT	141 (24.90)	[De Craeye *et al.*, 2008]
CZE	286	IFAT	126 (44.10)	[Dubey, 2010]
ESP	585	IFAT	189 (32.30)	[Miro *et al.*, 2004]
ESP	592	IFAT	103 (17.00)	[Montoya *et al.*, 2008]
FRA	52	MAT	33 (63.45)	[Afonso *et al.*, 2006]
HUN	330	IFAT	157 (47.60)	[Hornok *et al.*, 2008]

Table 3: Serological examinations of wild or stray cats for *T. gondii* antibodies in Europe (Spain). ESP: Spain; W: wild cat (*Felis silvestris*); L: lynx (*Lynx pardinus);* S: stray cat

Country	Species	No. samples analysed	Method	No. of positive samples (%)	Reference
ESP	S	317	IFAT	117 (36.90)	[Miro *et al.*, 2004]
ESP	W	12	IFAT	6 (50.00)	[Sobrino *et al.*, 2007]
ESP	L	27	IFAT	22 (81.50)	[Sobrino *et al.*, 2007]
ESP	L	26	IFAT	21 (80.70)	[Millan *et al.*, 2009b]
ESP	W	59	IFAT	50 (84.70)	[Millan *et al.*, 2009a]

Some studies show that experimentally infected cats may be immune to re-shedding of oocysts upon re-infection with *T. gondii* [Dubey, 1995; Frenkel and Smith, 1982; Lappin *et al.*, 1996]. However, [Dubey, 1996] showed that such immunity will decrease with age and that shedding of oocysts can occur after re-infection with *T. gondii* six years after the primary infection.

In cats, *T. gondii* infection rarely results in fatal or pronounced disease symptoms. The main symptoms of toxoplasmosis in cats after natural or experimental infection were reported to include interstitial pneumonia, hepatitis, splenitis, lymphoadenomegaly, and ophthalmitis [Dubey, 2005]. In Europe, only a few cases of symptomatic toxoplasmosis in cats were reported from Denmark, Italy and Switzerland [Anfray *et al.*, 2005; Henriksen *et al.*, 1994; Spycher *et al.*, 2011].

Contamination of food and water with oocysts is one of the main sources for human *T. gondii* infection. Warnings to pregnant women are issued in Germany and the EU to highlight this fact [Anonymous, 2007]. However, ingestion of raw or undercooked meat containing tissue cysts may also lead to infection with *T. gondii* [Dabritz and Conrad, 2010; Dubey and Jones, 2008; Dubey, 2010]. It has been argued that the parasite can also be transmitted via blood or leukocyte trans-

fusion [Tenter *et al.*, 2000]. Some of the toxoplasmosis outbreaks in Brazil and Canada could be traced back to contaminated water reservoirs [Bowie *et al.*, 1997; de Moura *et al.*, 2006]. A multicentre study including Belgium, Denmark, Italy, Norway and Switzerland identified ingestion of raw or undercooked meat such as lamb and beef, but also contaminated soil as main sources of *T. gondii* infections [Cook *et al.*, 2000]. In Norway, cured meat and poor kitchen hygiene were further significant risk factors for infection [Kapperud *et al.*, 1996]. Since the first successful isolation of *T. gondii* from humans and animals [Sabin and Olitsky, 1937] and the introduction of the dye test [Sabin and Feldman, 1948], *T. gondii* was recognised as a parasitic disease of animals and humans occurring worldwide. It is estimated that up to one third of the human population is infected with *T. gondii*. However, seroprevalences vary greatly between continents, geographical regions and ethnic groups [Tenter *et al.*, 2000]. Seroprevalence studies from Spain showed that 41.00% of migrant women had IgG anti-*T. gondii* antibodies [Ramos *et al.*, 2011], whereas only 12.00% of Spaniards tested positive for *T. gondii*.

In 2005, 1,519 (0.84 cases per 100,000 inhabitants) toxoplasmosis cases were reported to the European Centre for Disease Prevention and Control (ECDC) by 14 European countries. Of those countries, Lithuania and Slovakia reported the highest incidences with 6.86 and 4.84 per 100,000. Unfortunately, there is high variation between member countries in the criteria for reporting cases of clinical toxoplasmosis. Whereas in Norway only encephalitis cases need to be reported, Denmark also reports congenital cases from neonatal screening. Furthermore, only nine of the 25 EU member states submitted data to the ECDC in 2005. Together with data from the USA, a decrease in the *T. gondii* seroprevalence has been observed since 1999 [European Centre of Disease and Control, 2007; Jones *et al.*, 2009]. Like the seroprevalence in cats, *T. gondii* seroprevalence in humans is also shown to increase with age [Jones *et al.*, 2001]. There is very little information on the epidemiology of human toxoplasmosis in Germany, but a lot more research is undertaken in other European countries and the USA.

Generally, different forms of human toxoplasmosis can be observed. Congenital toxoplasmosis (CT) occurs when a woman becomes infected with *T. gondii* during pregnancy. This can result in a transplacental transmission of the parasite to the foetus leading to a wide range of clinical manifestations such as foetal death, hydrocephalus, intercerebral calcifications, mental retardations, or retinochoroiditis. However, infection can also be clinically unapparent. Some symptoms may only appear many years post-infection [Remington *et al.*, 2006]. Only recently, it was recognised that transmission of *T. gondii* to the foetus can not only occur during a primary infection during gestation but also due to a persistent parasitaemia from an infection contracted prior to conception but continuing well into the pregnancy [Lindsay and Dubey, 2011]. Furthermore, in HIV-infected or otherwise immunosuppressed mothers, re-activation may lead to vertical transmission of *T. gondii* [Azevedo *et al.*, 2010; Remington *et al.*, 2006]. After it had been discovered that different avirulent and virulent strains of *T. gondii* exist [Saeij *et al.*, 2006; Sibley and Boothroyd, 1992b], a further way of vertical transmission has been postulated to occur when an infection with a certain strain of *T. gondii* is followed by a secondary infection with a differing *T. gondii* strain [Elbez-Rubinstein *et al.*, 2009]. Extensive research was undertaken to assess the seroprevalence and risk of infection with *T. gondii* in pregnant women and women of child-bearing age; an overview of the results is shown in **Table 4**. It is estimated that three foetal losses per year are incurred by infection with *T. gondii* in the Netherlands [Hofhuis *et al.*, 2011; Kortbeek *et al.*, 2009]. However, the overall seroprevalence of *T. gondii* in pregnant women can be quite high. Depending on the country, seroprevalences can reach 50–77% as observed in Serbia and France [Baril *et al.*, 1999; Bobic *et al.*, 1998], respectively, or may be as low as 3% in Greece [Messaritakis *et al.*, 2008].

Table 4: Seroprevalence of *T. gondii* in pregnant women and women of childbearing age in Germany and Europe.

AUT: Austria; GER: Germany; DNK: Denmark; ESP: Spain; FRA: France; FIN: Finland; GRC: Greece; KAZ: Kazakhstan; NLD: Netherlands; NOR: Norway; SCG: Serbia; SWE: Sweden

Country	Sample size (n)	Prevalence (%)	Reference
GER	2,104	54.00	[Roos *et al.*, 1993]
GER	5,670	39.00	[Beringer, 1992]
GER	465	34−61.00	[Gross, 2004]
AUT	167,041	43.00	[Aspöck and Pollak, 1992]
DNK	89,875	25.30	[Lebech *et al.*, 1999]
ESP	1,488	12.00	[Ramos *et al.*, 2011]
ESP	1,627	41.50	[Ramos *et al.*, 2011]
FRA	13,459	54.30	[Baril *et al.*, 1999]
FIN	16,733	20.30	[Lappalainen *et al.*, 1995]
GRC	9,285	3.10	[Messaritakis *et al.*, 2008]
GRC	1,157	40.00	[Diza *et al.*, 2005]
KAZ	3,126	16.00	[Torgerson *et al.*, 2009]
Kosovo	334	29.40	[Dentico *et al.*, 2011]
NLD	7,521	40.50	[Kortbeek *et al.*, 2004]
NOR	35,940	10.90	[Jenum *et al.*, 1998]
SCG	1,197	77.00	[Bobic *et al.*, 1998]
SWE	35,000	10.10	[Evengard *et al.*, 2001]

However, one has to be careful with the interpretation of the results from Greece since only the inhabitants of the islands of Crete and Cyprus were examined and the low seroprevalence was and attributed to an increased consumption of frozen food stuff. A more comprehensive study examined 603 confirmed cases of *T. gondii* infections during pregnancy in France. The transmission of *T. gondii* was 26.70% (161/603). In eight cases, vertical transmission resulted in either still birth (3) or abortion (5) of the foetus. The remaining 153 births were followed up regularly and in 41 cases (26.80%) clinical signs were observed later in life. They

included retinochoroiditis (33), intracerebral calcification (14) or a combination of the two clinical symptoms (4) [Dunn *et al.*, 1999].

Overall, the *T. gondii* prevalence is estimated as 1–10 births per 10,000 live births [Cook *et al.*, 2000; Evengard *et al.*, 2001]. A study of an acute toxoplasmosis outbreak in Canada showed that the prevalence of *T. gondii* was 0.90% in pregnant women [Burnett *et al.*, 1998]. As for cats, a correlation of age and seroprevalence with respect to *T. gondii* was recognized in humans. Data from Kazakhstan, the Netherlands and Italy confirmed that with increasing age, the risk of acquiring *T. gondii* increased as vertical transmission did during pregnancy by the parasite [Buffolano *et al.*, 1996; Kortbeek *et al.*, 2004; Torgerson *et al.*, 2009]. In Poland, 17,653 filter paper samples from live-born neonates were successively screened for *T. gondii* and 19 new-borns (0.11%) were seropositive [Paul *et al.*, 2001]. In Europe, different treatment regimes are available when congenital toxoplasmosis is detected, consisting of the application of spiramycin, pyrimethamine-sulphonamide, sulfadoxine and pyrimethamine (Fansidar) depending on the trimester of pregnancy [Christoph *et al.*, 2004]. While an outbreak of toxoplasmosis in Canada was relatively benign, another one in French Guiana resulted in one death and two cases of lethal congenital toxoplasmosis. It was postulated that at least 5 of 11 clinical cases observed during this outbreak in French Guiana were caused by the same *T. gondii* strain. However, the isolated *T. gondii* strain was different from strains observed in Europe or the USA [Demar *et al.*, 2007].

Ocular toxoplasmosis (OT) can occur after postnatal infection or as a consequence of a congenital infection. Most epidemiological research concludes that postnatal infection is the main cause of ocular toxoplasmosis rather than congenitally acquired infection [Gilbert and Stanford, 2000; Holland, 1999, 2000]. *T. gondii* tachyzoites can reach the eye via the blood stream and are arrested in the capillary bed of the retina [Roberts and McLeod, 1999]. Within the retina, the parasite multiplies causing cell lysis and lesions. Reactivation may also be followed by the occurrence of re-newed ocular toxoplasmosis [Burnett *et al.*, 1998].

Since ocular diseases were also identified in foetuses, ocular toxoplasmosis may also be caused *in-utero*. Although most symptoms are the result of damage by the parasite, a pronounced Th1 immune response, especially high concentrations of IL-6, TNF-α and IFN-γ were discovered in patients with retinochoroiditis [Montoya and Liesenfeld, 2004; Pleyer *et al.*, 2009; Torun *et al.*, 2002]. Clinical symptoms include decrease of visual acuity, pain in the eye and photophobia. Retinal lesions may form and thus reduce vision. The extent of damage will depend on the location and size of the lesion, as well as the extent of retinal inflammation as a response to the parasite [Atmaca *et al.*, 2004; Bosch-Driessen *et al.*, 2002a; Bosch-Driessen *et al.*, 2000; Bosch-Driessen *et al.*, 2002b; Munoz *et al.*, 2011]. In a study of 14 patients with acquired OT, in 54.00% of the patients reactivation occurred within two years [Bosch-Driessen and Rothova, 1999; Bosch-Driessen *et al.*, 2002b]. The best described cases were the result of an acute toxoplasmosis outbreak in Canada. A total of 100 patients with acquired and 12 patients with congenital toxoplasmosis were identified. Twenty patients showed evidence of retinal infection whereas no alterations were found in the remaining patients. No gender differences were observed and all age groups (15–83 years) were affected. Most cases presented unilateral damage to the eye with single retinal lesions and poor levels of acuity were observed in patients with macular lesions or significant vitris. In some cases, depending on the location and kind of damage, vision may be improved by administration of anti-inflammatory drugs such as clindamycin or prednisone. It was estimated that 0.26–0.69% of infected individuals presented with retinitis at the outbreak [Burnett *et al.*, 1998]. Another study followed 25 children with diagnosed congenital toxoplasmosis. Eighteen children (72.00%) developed at least one lesion, 13 children (52.00%) new central and 11 children (44.00%) peripheral lesions. Fifty-two per cent had new (activated) lesions after 10 years [Phan *et al.*, 2008]. Some researchers suggest that certain unusually virulent strains of *T. gondii* are the prime cause of OT in the US and South America, where OT is a big problem in parts of Brazil [de Moura *et al.*, 2006; Khan *et al.*, 2006b; Silveira *et al.*, 2001; Vaudaux *et al.*, 2010]. A comparative study of OT in

Brazil and Europe concluded that children in Brazil developed retinochoroidal lesions earlier. They had a greater risk of multiple recurrences and large lesions than European children [Gilbert *et al.*, 2008].

The immune status of infected patients is also of importance for the outcome of the disease. More severe complications, such as encephalitis and OT were observed in HIV-infected patients [Luft and Remington, 1992; Mariuz and Luft, 1992; Rabaud *et al.*, 1994]. Other cases of cerebral toxoplasmosis were observed in patients with common variable immunodeficiency (CVID) ([Holtkamp *et al.*, 2004] and in transplantation patients ([Hommann *et al.*, 2002]. Again, in such patients high prevalences of virulent *T. gondii* strains were observed [Khan *et al.*, 2005a].

Behavioural disorders represent another feature of toxoplasmosis. Many studies report a higher *T. gondii* prevalence in patients with schizophrenia compared with control patients [Alvarado-Esquivel *et al.*, 2011; Arling *et al.*, 2009; Fekadu *et al.*, 2010; Niebuhr *et al.*, 2008; Xiao *et al.*, 2009]. Individuals with latent *T. gondii* infections show different personality profiles than those without infection. Studies from the Czech Republic suggest that infected men had a lower rule consciousness and were more likely to be jealous whereas the opposite was true for *T. gondii* infected women [Flegr and Havlicek, 1999; Flegr, 2010]. In animals infected with *T. gondii,* the risk perception and novelty seeking is increased [House *et al.*, 2011; Vyas *et al.*, 2007; Webster *et al.*, 2006; Webster, 2007]. However, whether *T. gondii* is the prime cause of such condition remains controversial since other studies found no correlation between seroprevalence and schizophrenia [Hinze-Selch *et al.*, 2007; Hinze-Selch *et al.*, 2010]. Different mechanisms have been postulated to explain these conditions which include cyst formation in certain areas of the brain or induction of host immune responses. However, psychiatric disorders were not shown to be more prevalent in areas with a high *T. gondii* seroprevalence [Fekadu *et al.*, 2010].

1.4 *T. gondii* population structure and genotypes

As already mentioned, *T. gondii* has not only the capacity to propagate asexually but also sexually in its feline definitive host. Therefore sexual recombination should provide for a high genetic diversity between *T. gondii* strains worldwide. In contrast to this hypothesis, only a small number of *T. gondii* strains dominate in North America and Europe. First evidence of the existence of different strains came from isoenzyme analysis of 35 French isolates [Darde *et al.*, 1992]. It was hypothesised that only three types of *T. gondii* existed, a hypothesis that was later confirmed with the advent of genetic analysis techniques, such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). *T. gondii* from North America and Europe fell into three genotypes, termed type I, type II and type III [Howe and Sibley, 1995; Sibley and Boothroyd, 1992b]. Interestingly, type I was more virulent in mice than types II or III [Howe *et al.*, 1996]. Analysing individual genes, such as GRA6 showed little variation between the isolated types [Fazaeli *et al.*, 2000; Grigg and Boothroyd, 2001]. However, in these early studies only a limited number of loci (markers or genes) were analysed, so that the first South American isolates from chicken also fell into one of those three distinct types [Dubey *et al.*, 2002]. Interestingly, microsatellite analysis of different types identified highly polymorphic loci due to its higher resolution [Ajzenberg *et al.*, 2002a; Blackston *et al.*, 2001] leading to the hypothesis that all three types might have had a recent origin. Large scale sequencing of Expressed Sequence Tags (ESTs) discovered new genes and Single Nucleotide Polymorphisms (SNPs). These are small changes in the nucleotide sequences between different types of *T. gondii* [Ajioka *et al.*, 1998; Manger *et al.*, 1998]. Using sequencing techniques, the first analysis of North American and Brazilian *T. gondii* strains discovered a different genetic composition of South American isolates. Such genetic differences were believed to be the reason for the unusually high frequency of severe ocular toxoplasmosis in Brazil. Furthermore, the linkage disequilibrium was high in North American strains which indicated a higher rate of selfing (asexual propagation). In contrast, the low linkage disequilibrium in

Brazilian strains was believed to be the result of higher rates of transmission and thus higher rates of outcrossing event (sexual recombination) [Lehmann *et al.*, 2004]. Microsatellite analysis of South American isolates showed, for the first time, highly diverse and distinct patterns from European and North American *T. gondii* [Ajzenberg *et al.*, 2004]. A major break-through included the generation of genetic crosses between different types of *T. gondii* using drug resistant strains. Analysing the crosses of type II and type III [Sibley and Boothroyd, 1992a], type I and type III [Su *et al.*, 2002] as well as newly identified markers led to the first composite genetic map of *T. gondii* which provided the framework for further genetic studies. It not only identified three new chromosomes, bringing the total number of chromosomes (Chr.) to 14 (Chr. Ia, Ib, II, III, IV, V, VI, VIIa, VIIb, VIII, IX, X, XI and XII) but also showed that several chromosomes showed homologous type specific patterns. Chr. IV was shown to have exclusively type II specific SNPs whereas Chr. XI consisted primarily of type I specific SNPs [Khan *et al.*, 2005b]. Further analyses of genetic sequences of different *T. gondii* strains from humans and animals from France and the USA discovered that Chr. Ia was shared between all type I and type II *T. gondii* [Khan *et al.*, 2006a]. Closer analysis of Chr. Ia discovered that this Chr. was very similar or identical in all three types and thus inherited in common by all three types about 10,000 years ago [Su *et al.*, 2006]. Furthermore, 4,324 SNPs were identified among the three types, using new EST data. It became clear that large chromosomal regions were dominated by one of the three type specific SNPs. For example 98.00% of Chr. XI contained type I SNPs whereas 90.00% of Chr. IV was dominated by type III SNPs. Since some Chr. (except for Chr. Ia) contained only a maximum of two different type specific SNPs and clear transition points between those specific SNPs existed, it was hypothesised that the present *T. gondii* types must have originated by recombination of only a few of ancestral crosses. The fact that polymorphic patterns between type I and type III SNP regions and high levels of atypical SNPs in type II dominated region existed lead to the theory that two separate crossing event between ancestral versions of the present type II and two distinct strains

must have occurred. A diagram of the proposed models is shown in **Figure 2**. According to this model, type II parental strains (which are not very similar to each other) are divergent from strain α and strain β. Strains α and β are distinct from each other but closer related to each other than to type II. According to this model, a cross between an ancestral type II and α led to the modern-day type I and a cross between an ancestral type II and β produced the modern-day type III. A type II ancestor similar to the other type II ancestors leading to the emergence of modern-day types I and III was involved in the emergence of the present-day type II strains. Due to the criteria of the chromosomal SNP patterns under such model, the isolate P89 was postulated to be strain β. [Boyle *et al.*, 2006]. Newly discovered loci lead to a widespread use of nine PCR-RFLP markers, called newSAG2 (Chr. VII), SAG3 (Chr. XII), BTUB (Chr. IX), GRA6 (Chr. X), c22-8 (Chr. Ib), c29-2 (Chr. III), L358 (Chr. V), PK1 (Chr. VI) and Apico (extra chromosomal) for genotyping *T. gondii* isolates worldwide. Genotyping progressed from looking at only one or four single loci to analysing nine unlinked, independent loci thus increasing resolution [Su *et al.*, 2006].

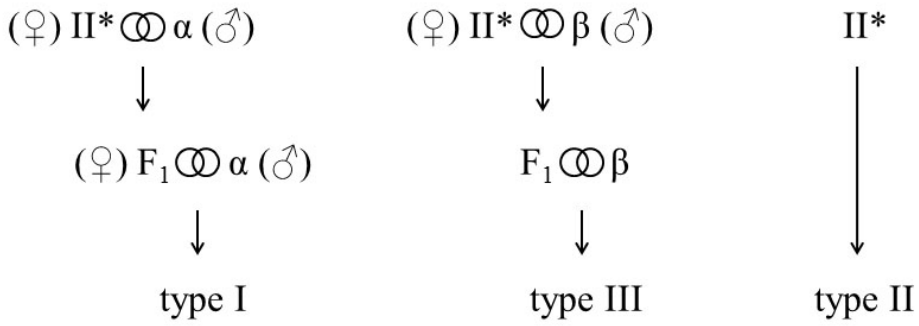

Figure 2: Proposed model of inheritance creating the present-day *T. gondii* genotypes.
[Boyle *et al.*, 2006]-modified

The isolation of more South American strains from Brazil showing a PCR-RFLP pattern previously not observed in types I, II and III, termed “atypical” isolates facilitated in depth research into the population structure of *T. gondii* [Su *et al.*, 2006]. By analysing 275 isolates from free-ranging chickens collected from all over the world, the first comprehensive population study was conducted using five Short Tandem Repeats (STRs) loci, one microsatellite and the SAG2 locus. In this study, two South American populations of high genetic diversity were identified, as well as extensive genetic differences in *T. gondii* isolates from the new and the old world. While North American isolates closely resembled the European isolates (old world), African isolates clustered in between old and new world isolates. Using a bayesian statistical model, i.e. the program STRUCTURE, only four populations of *T. gondii* were identified. Two genetically diverse populations were confined to South America (SA1 and SA2), another population showing small genetic differences was situated in Europe, North America, Asia and Africa (RW) whereas the fourth population showed cosmopolitan abundance (WW). Some strains spread globally within a short period of time as evident from mutational distances among them. Therefore long-term isolation and extensive migration of *T. gondii* must have played major roles. South America was concluded to be the birthplace of modern-day *T. gondii* strains followed by two migration events into North America and Europe. One migration event must have taken place in Eurasia possibly mediated by man. Ships, carrying *T. gondii* infected intermediate host species such as mice and rats into Eurasia have been proposed as one possible route. The second migration event was postulated to have occurred as part of the global maritime sea trade which included food stuff, livestock, cotton and slaves. This could have dispersed the RW population to other continents where no *T. gondii* population was established at that time. Increased trade was proposed to have led to the establishment of the WW population even into areas that were already inhabited by *T. gondii* [Lehmann *et al.*, 2006]. Interest in the population structure of *T. gondii* grew with the identification of new South American isolates and new typing methods. By analysing the frequencies of SNPs within eight introns in five

unlinked loci and Chr. Ia, it was further confirmed that there was segregation between strains from South America and Europe/North America. All 46 strains used in the study were grouped into 11 distinct haplogroups. Haplogroups 1–3 (containing types I, II and III, respectively) originated exclusively from Europe and North America whereas haplogroups 4, 5 and 8–10 occurred only in South America. Haplogroup 6, however, was found to be wide spread in Europe, North America, South America and Africa. Genotypes that were termed "atypical" because they showed an unknown PCR-RFLP pattern at some loci were found to be common in South America. Collectively, the data suggested that European/North American strains diverged from South American strains approximately 1,000,000 years ago, but that a small number of types (I, II and III) must have rapidly expanded within the past 100,000–10,000 years. It was postulated that an admixture of four ancestral lineages, similar to haplogroups 2, 4, 6 and 9 had resulted in the 11 haplogroups. If the apicoplast, a secondary endosymbiont that is only inherited maternally [Ferguson *et al.*, 2005] is taken into account, haplogroups 1, 2, 4 and 8 must have derived from a different matrilineage than haplogroups 3, 6 and 9 [Khan *et al.*, 2007]. Closer examination of South American isolates confirmed that such atypical strains were genetically highly diverse and common in Brazil. It was also discovered that within South America some strains were more common than others and formed distinct clonal groups different from types I, II and III that appeared in North America and Europe. The Brazilian clonal types were termed BrI, BrII, BrIII. Even more interesting members within the clonal groups showed different virulence in mice. Type BrI was highly mouse-virulent and truly "atypical", whereas types BrII and BrIII were non-virulent in mice and showed a combination of alleles specific for types I, II and III at different loci. Such strains are now referred to as non-canonical *T. gondii* and were postulated to have recently expanded in South America [Pena *et al.*, 2008].

Other genotypes, different from types II and III were also discovered in Africa [Velmurugan *et al.*, 2008]. The discovery of a *T. gondii* strain from Uganda showed that recombination events between *T. gondii* strains have occurred in Africa. In addition new SNPs, i.e. SNPs not common in European and North American strains, were identified in Ugandan type II (UgII) and type III Ugandan (UgIII) strains. Furthermore, it was shown that such type III isolates from Uganda differ in their virulence from European/North American type III. SNP data suggested that the age of the most recent ancestor (MRCA) of UgII and type II (Europe) was 17,400 years, whereas the divergence of the UgII isolate from type III was 9,200 years. UgII was found to be more divergent from the European and North American type II than UgIII from the European and North American type III. The recombinant strain TgCkUg2 arose by chromosomal sorting rather than interchromosomal recombination [Lindström Bontell *et al.*, 2009]. New African haplogroups were identified in Gabon by microsatellite analysis using 13 markers. Cluster analysis resulted in 3 groups: group 1 included an Africa 1 haplogroup together with type I, group 2 included an Africa 3 haplogroup and group 3 all type III/type III-like strains [Mercier *et al.*, 2010]. Even more interesting, *T. gondii* isolated from wild animals from French Guiana were highly divergent, whereas isolates from domestic animals in the same country in an anthropised environment shared their genotypes. Except for one isolate, a clear boundary was observed between wild and anthropised isolates indicating that two distinct *T. gondii* populations existed which correspond to the domestic and the wild cycle of *T. gondii* [Mercier *et al.*, 2011].

The current population structure of *T. gondii* suggests strong geographic segregation between the European/North American strains and the South American strains [Khan *et al.*, 2011b]. It also includes the newly diverged African and newly identified Chinese [Zhou *et al.*, 2010] genotypes. All strains included in this new analysis were characterised by sequencing eight introns on Chr. IV, VIIa, IX, X and XI. Fourteen haplogroups were described in total forming 4 major clus-

ters. Cluster 1 includes haplogroups 1–3, 7, 11 and 12 those isolates are found in Europe/North America. Cluster 2 includes haplogroups 4, 5, 6, 8, 9, 10 that contain isolates from South America as well as Africa. The last two clusters consist of haplogroup 13 and 14 that include parasites isolated from China and Africa, respectively. Interestingly haplogroups 1, 2, 3, 4, 7, 8, 9 and 13 were found to be highly similar for Chr. Ia. This monomorphic Chr. Ia will be referred to as Chr. Ia* in the following. Interestingly, haplogroups 5 and 10 (containing South American and African isolates) were shown to possess completely different versions of Chr. Ia in each isolate. Haplogroup 13 (Chinese isolates) contained only few SNPs divergent from Chr. Ia* and also clustered with other Chr. Ia haplogroups. Regarding Chr. Ia, haplogroup 6 clustered with haplogroup 14 (African isolates) and had a Chr. Ia divergent from Chr. Ia* at the 3' end (3' chimeric Chr. Ia), whereas haplogroups 11 and 12 (isolated from wild animals from North America) showed a Chr. Ia divergent from Chr. Ia* at the 5' end (5' chimeric Chr. Ia) [Khan *et al.*, 2011b]. Interestingly, haplogroup 12 had only been discovered recently as a fourth clonal lineage in North America and was found to be restricted to wild animals [Khan *et al.*, 2011a]. Analysis of Chr. Ia of haplogroups 11 and 12 indicated a single meiotic recombination in the wild. According to the SNPs found in intron sequences, North American/European *T. gondii* split from South American lineages approximately 100,000 years ago, while North American and European strains separated approximately 10,000 years ago. Haplogroup 12 is postulated to be the most likely parent that led to the type II observed today. Large blocks of type III SNPs on Chr. Ia were shared in haplogroup 9 whereas large blocks of type I SNPs were shared on Chr. Ia in haplogroup 6. The age of the respective groups are consistent with the hypothesis that ancestors of haplogroup 9 led to the formation of haplogroup 3 (type III) and that ancestors of haplogroup 6 led to the formation of haplogroup 1 (type I). Therefore ancestral members of haplogroup 9 (e.g. the P89 isolate) could be the proposed factor ß and ancestral isolates of haplogroup 6 (e.g. the FOU isolate) might be closely related to the ancestral strain α providing the ancestral source of Chr. Ia to the now established type I. An overview of the hap-

logroups under the proposed model of Chr. Ia is given in **Table 5** [Khan *et al.*, 2011b].

As already mentioned, North America and Europe are dominated by types I, II, III. Although all three clonal types are observed in Europe to a certain extent, not all types are evenly distributed or found in all countries and in all infected species. In Germany, most isolates found until 2007 were derived from feline faecal samples but were almost exclusively of type II [Schares *et al.*, 2008b]. Type I was not detected when 20,317 feline faecal samples were examined. In fact, most isolates obtained until 2007 showed a high prevalence of genotype II in Europe. In Austria, 830 chickens were analysed and 67 *T. gondii* isolates recovered by bioassay in mice. Genotyping performed only at the SAG2 locus, resulted in *T. gondii* of type II in all cases [Dubey *et al.*, 2005a]. In the arctic (Norway), the majority of analysed foxes were found to be infected with *T. gondii* type II. Only four foxes (7.30%) were infected with type III, while type I was not found. However, 40.00% of *T. gondii*-DNA from foxes could not be fully genotyped and the *T. gondii* of two (3.60%) foxes shared more than one allele at a given locus or showed different type-specific alleles over ten loci [Prestrud *et al.*, 2008]. Furthermore, a number of *T. gondii* isolates from Portugal and Spain showed a high proportion of *T. gondii* type III [Dubey *et al.*, 2006e; Montoya *et al.*, 2008; Waap *et al.*, 2008]. In contrast, *T. gondii* isolated from chickens in Poland showed that atypical *T. gondii* were found in Europe [Dubey *et al.*, 2008b]. Genotyping results of *T. gondii* from animals in Europe are summarised in **Table 6.**

Table 5: Haplogroups of *T. gondii* isolated world-wide.

[Khan *et al.*, 2011b] - modified.

Eur: Europe; N-A: North America; S-A: South America; Afr: Africa; As: Asia; a: factor α; b: factor β; c: ancestral type II

Haplo-group	**Continent**	***T. gondii* strains (examples)**	**Chr Ia***	**3'Chr Ia***	**5'Chr Ia***	**Divergent**
1	Eur, N-A	type I	Yes			
2	Eur, N-A	type II	Yes			
3	Eur, N-A	type III	Yes			
4	S-A	MAS	Yes			
5	S-A	Guy				Yes
6[a]	Eur, N-A, S-A, Afr	FOU, Africa 1 isolates		Yes		
7	Eur, N-A	CAST	Yes			
8	S-A	TgCatBr6	Yes			
9[b]	Eur, N-A, S-A	P89, TgCatBr10	Yes			
10	S-A	VAND				Yes
11	N-A	TgCgCa1			Yes	
12[c]	Eur, N-A	TgSoUs13	Yes		Yes	
13	As	TgCtPRC2	Yes			
14	Afr	Africa 3 isolates		Yes		

Table 6: *T. gondii* isolates from animals in Germany and other countries in Europe until 2008.

AUT: Austria; DNK: Denmark; ESP: Spain; FRA: France; GER: Germany; ITA: Italy; NOR: Norway; POL: Poland; POR: Portugal; *: number of loci examined

Country	Type I (%)	Type II (%)	Type III (%)	Atypical (%)	Species	Method	Reference
GER	0/22 (0)	22/22 (100)	0/22 (0)	0/22 (0)	Cat	RFLP (4*)	[Schares *et al.*, 2008b]
ESP	4/26 (15)	22/26 (85)	0/26 (0)	0/26 (0)	Cat	RFLP (1)	[Montoya *et al.*, 2008]
DNK	0/36 (0)	36/36 (100)	0/36 (0)	0/36 (0)	Cat	mAb	[Jensen *et al.*, 1998b]
AUT	0/19 (0)	0/19 (0)	0/19 (0)	0/19 (0)	Chicken	RFLP (9)	[Dubey *et al.*, 2005a]
ITA	0/3 (0)	3/3 (0)	0/3 (0)	0/3(0)	Chicken	RFLP (9)	[Dubey *et al.*, 2008b]
POL	0/2 (0)	0/2 (0)	0/2 (0)	2/2 (100)	Chicken	RFLP (9)	[Dubey *et al.*, 2008b]
POR	0/12 (0)	8/12 (67)	4/12 (33)	0/12 (0)	Chicken	RFLP (1)	[Dubey *et al.*, 2006e]
NOR	0/55 (0)	27/55 (49)	4/55 (7)	2/55 (4)	Fox	RFLP (9)	[Prestrud *et al.*, 2008]
POR	1/12 (8)	9/12 (75)	2/12 (17)	0/12 (0)	Pigeon	MS (5)	[Waap *et al.*, 2008]
POR	0/15 (0)	11/15 (73)	4/15 (27)	0/15 (0)	Pig	RFLP (1)	[Sousa *et al.*, 2006]
FRA	0/8 (0)	8/8 (100)	0/8 (0)	0/8 (0)	Sheep	MS (5)	[Dumetre *et al.*, 2006]
UK	0/13 (0)	13/13 (100)	0/13 (0)	0/13 (0)	Sheep	RFLP (1)	[Owen and Trees, 1999]

Table 7: *T. gondii* isolates from human clinical cases with congenital toxoplasmosis in Europe until 2008.

ESP: Spain; FRA: France; POL: Poland; UK: United Kingdom; *: number of loci examined

Country	Type I (%)	Type II (%)	Type III (%)	Atypical (%)	Method	Reference
ESP	6/9 (75)	1/9 (13)	1/9 (13)	6/9 (75)	RFLP (1*)	[Fuentes *et al.*, 2001]
FRA	3/14 (21)	11/14 (79)	0/14 (0)	0/14(0)	MS (4)	[Costa *et al.*, 1997]
FRA	0/13 (0)	13/13 (100)	0/13 (0)	0/13 (0)	RFLP (1)	[Howe *et al.*, 1997]
FRA	7/86 (8)	73/86 (85)	2/86 (2)	4/86 (5)	MS (5)	[Ajzenberg *et al.*, 2002b]
POL	0/9 (0)	9/9 (100)	0/9 (0)	0/9 (0)	RFLP (5)	[Nowakowska *et al.*, 2006]
UK	6/19 (32)	7/19 (37)	0/19 (0)	0/19(0)	RFLP (1)	[Aspinall *et al.*, 2003]

Table 8: *T. gondii* isolates from immunosuppressed human clinical cases with toxoplasmosis in Europe until 2008.

ESP: Spain; FRA: France; UK: United Kingdom ; *: number of loci examined

Country	Type I (%)	Type II	Type III (%)	Atypical (%)	Condition	Method	Reference
ESP	8/31 (26)	18/31 (58)	5/31 (16)	0/31 (0)	HIV	RFLP (1*)	[Fuentes *et al.*, 2001]
FRA	7/55 (13)	41/55 (75)	0/55 (0)	0/55 (0)	HIV	RFLP (1)	[Honore *et al.*, 2000]
FRA	6/45 (13)	34/34 (76)	5/45 (11)	0/45 (0)	HIV	RFLP (1)	[Howe *et al.*, 1997]
UK	4/8 (50)	1/8 (13)	0/8 (0)	0/8 (0)	HIV	RFLP (1)	[Aspinall *et al.*, 2003]
FRA	3/16 (19)	12/16 (75)	1/16(6)	0/16 (0)	non-HIV	RFLP (1)	[Honore *et al.*, 2000]
FRA	1/10 (10)	8/10 (80)	1/8 (10)	0/8 (0)	non-HIV	RFLP (1)	[Howe *et al.*, 1997]
ESP	4/17 (24)	9/17 (53)	4/17(24)	0/17 (0)	unknown	RFLP (1)	[Fuentes *et al.*, 2001]

Few analyses of human congenital toxoplasmosis exist for Europe. They showed that the majority of clinical cases are caused by *T. gondii* of type II but that type I and type III have nevertheless been found in humans all over Europe [Ajzenberg *et al.*, 2002a; Aspinall *et al.*, 2003; Fuentes *et al.*, 2001; Honore *et al.*, 2000; Howe *et al.*, 1997; Nowakowska *et al.*, 2006] as shown in **Table 7**. In human clinical cases with immunosuppression, similar genotypes prevail. However, in contrast to congenital human cases, more type I and type III *T. gondii* were observed in these patient groups [Aspinall *et al.*, 2003; Fuentes *et al.*, 2001; Honore *et al.*, 2000; Howe *et al.*, 1997] as shown in **Table 8**.

Regarding *T. gondii* genotypes associated with ocular toxoplasmosis, very few studies exist. Using five loci (SAG1, SAG2, SAG3, SAG4 and B1) to analyse twelve clinical cases of ocular toxoplasmosis in the USA, three cases were identified as *T. gondii* type I, three were of type II and only one was of type III. The five remaining *T. gondii* isolates were of non-canonical or atypical genotypes [Grigg *et al.*, 2001b]. Genotyping of *T. gondii* isolated from a Korean patient with ocular toxoplasmosis identified *T. gondii* type I [Quan *et al.*, 2008]. SNP analysis of a repetitive polymorphic region of the non-transcribed spacer 2 (NTS2) located between the 28S and 18S rRNA genes demonstrated that all from Poland were infected with *T. gondii* of type I [Switaj *et al.*, 2006]. However, since comparisons with more established genotyping techniques were not carried out, it is difficult to interpret the results of this study. Unfortunately, no studies exist on *T. gondii* genotypes in humans from Germany.

Compared to South America, the genetic diversity of *T. gondii* in animals and humans in Europe and North America is low. Genotyping of animals in South America revealed that the majority of *T. gondii* isolates from free-ranging chickens in Nicaragua, Brazil, Chile, Colombia and Guyana harboured a high number of non-canonical and atypical *T. gondii* [Dubey *et al.*, 2005b; Dubey *et al.*, 2006b; Dubey *et al.*, 2006d; Dubey *et al.*, 2007a; Dubey *et al.*, 2008e]. Other genetically different non-canonical and atypical *T. gondii* were observed in dogs from Vi-

etnam, Colombia and Sri Lanka [Dubey *et al.*, 2007b; Dubey *et al.*, 2007c; Dubey *et al.*, 2007d; Dubey *et al.*, 2007e] as well as cats from China [Dubey *et al.*, 2007h] and Brazil [Pena *et al.*, 2008]. Other atypical *T. gondii* isolated were observed in a toxoplasmosis outbreak in Surinam and in wild jaguars in French Guiana [Demar *et al.*, 2007; Demar *et al.*, 2008]. Genotypic analysis of immunocompromised humans with toxoplasmosis from Brazil using three loci revealed that 40/87 (45.98%) patients were infected with *T. gondii* type I, 9/87 (10.35%) with type II and 4/87 (4.60%) with type III. The remaining patients showed *T. gondii* of a polymorphic allele pattern (19/87; 21.83%) or their genotype could not be determined (15/87; 17.24%) [Ferreira *et al.*, 2008; Ferreira *et al.*, 2011].

In contrast, *T. gondii* observed in pigs and sheep from the USA were mostly of type II or III and only a few *T. gondii* showed a non-canonical or atypical allele pattern [Dubey *et al.*, 2008a; Dubey and Jones, 2008; Dubey *et al.*, 2008d]. Surprisingly, *T. gondii* from wild mammals, such as skunk, black bear, racoon and sea otters from the USA were shown to be of non-canonical types [Dubey *et al.*, 2008c; Miller *et al.*, 2008].

1.5 *T. gondii* virulence and virulence factors

First experiments with *T. gondii* types I, II and III showed that type I was highly virulent for outbred mice. Acute infection with type I (RH) was not dose-dependent and resulted always in the death of all infected mice. The lethal dose (LD), which resulted in 100 per cent death in a group of mice, was equal to one parasite ($LD_{100} = 1$). In contrast, infection with type II (ME49) or III (CEP, NED) had a reduced virulence with an $LD_{50} > 10^3$ parasites [Howe *et al.*, 1996; Sibley and Boothroyd, 1992b]. Infection with *T. gondii* regardless of the type was shown always to result in a loss of weight in mice. However, the onset of the response was different depending on the dose and the genotype of *T. gondii*. Infection by

clonal type I resulted in an earlier and more pronounced weight loss than observed in mice infected with types II or III. Also, mice infected with types II or III always showed a period of weight gain after initial weight loss, but never reached their pre-infection weight [Jensen *et al.*, 1998a]. Most data on virulence was established in mice. However, a *T. gondii* of type III (O14) isolated from abortive sheep showed no adverse effects in mice but resulted in high virulence when used for infection in pigs [Jungersen *et al.*, 1999; Jungersen *et al.*, 2002]. Thus, virulence seems be influenced by both host and parasite factors. In contrast, most humans infected in Europe were shown to be infected with *T. gondii* type II and only a few infections with type III were reported (see previous section). It was therefore argued that human *T. gondii* infections simply reflect the presence of *T. gondii* genotypes in the respective animal reservoir [Boothroyd and Grigg, 2002].

Interestingly, most type I *T. gondii* strains used to date in experimental studies were isolated from a humans (RH) or goats (GT1) a number of decades ago. It has therefore been suggested that continued laboratory passage of these strains, e.g. the RH strain, may have led to increased virulence. However, it was shown that all different RH strains existing in laboratories with varying passage numbers resemble in their virulence pattern to mice [Su *et al.*, 2002] and possess similar growth properties and gene expression levels. Other researchers observed differences in the multiplication and growth rate between the different types of *T. gondii*. Again, type I showed increased doubling times as compared to types II and III [Radke *et al.*, 2001]. Enhanced migration, together with higher growth rates, leading to increased dissemination, a more rapid growth and a higher tissue burden, induced proinflammatory mediators resulting in more pronounced pathology [Barragan and Sibley, 2002; Barragan *et al.*, 2005; Gavrilescu *et al.*, 2004].

Immunological investigations revealed that *T. gondii* elicits a Th1 response during and after entry of *T. gondii* into host cells. This response is characterised by an increased interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin 12 (IL-12) release by antigen-presenting cells (APCs) such as dendritic

cells**.** Upon infection with *T. gondii*, APCs recognise surface antigens via toll-like receptors (TLRs) and pathogen associated molecular patterns (PAMPs). Activation of such receptors starts a cascade that culminates in the activation and translocation of nuclear factor-kappa B (NF-kB) to the nucleus of the APC and transcription of pro-inflammatory responses such as IL-12. Released IL-12 can activate other immune cells such as natural killer cells (NKs) or T-cells leading to a secretion of further inflammatory mediators such as IFN-γ. IFN-γ can then bind to its receptors on infected cells activating the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, leading to the phosphorylation of STAT1 which is translocated to the nucleus. This leads to the transcription of IFN-γ-stimulated genes such as interferon regulated factor 1 (IRF1), class II Major Histocompatibility Complex (MHC) and interferon regulated GTPases (IRGs). IRGs were shown to accumulate on the PVM in infected cells, leading to IRG-dependent killing of *T. gondii* [Melo *et al.*, 2011].

The process of translocating NF-kB to the host cell nucleus was shown to be strain-dependent. While infection with *T. gondii* types II and III led to a translocation of NF-kB, infection with virulent *T. gondii* type I did not. Furthermore, IL-12 release seemed to be initiated by type II, but not by type I. However, antigens isolated from the respective types alone did not elicit any IL-12 release in neutrophils. Therefore, an active penetration or infection was needed to elicit any host cell response [Denkers *et al.*, 2004; Robben *et al.*, 2004].

After the first genetic crosses between types II and III, as well as between types I and III had been generated [Grigg *et al.*, 2001a; Su *et al.*, 2002], mouse virulence and its determinants were investigated. A cross between the two avirulent *T. gondii* types II (PTG) and III (CTG) surprisingly revealed that offspring of the F1 generation was of higher virulence in mice than either of the parental clones [Grigg *et al.*, 2001a]. The same phenomenon was observed in crosses between the virulent type I (GT-1) and the avirulent type III (CTG). This cross allowed to identify quantitative trait loci (QTL) associated with an increased viru-

lence in the progeny. QTL-analysis revealed that an increased virulence was strongly linked to a region on Chr. VII with a minor contribution from Chr. Ib and IV [Su *et al.*, 2002]. Genetically engineered strains of the *T. gondii* type IIxIII cross expressing luciferase confirmed that the more virulent strains had a higher growth rate in mice and disseminated better in mouse tissue as compared to less virulent strains [Saeij *et al.*, 2005].

The progeny of crosses between the respective types were further assessed by QTL-analysis of total RNA isolated from the progeny against a cDNA library of type I (RH). Applying 175 genetic markers used for mapping the genome [Khan *et al.*, 2006a] identified five virulence regions associated with virulence in the *T. gondii* progeny. The VIR1 region included the candidate genes ROP5 and SAG3 on Chr. XII, while VIR2 indicated that unidentified genes on Chr. X were linked to virulence. VIR3 identified ROP18 on Chr. VIIa, VIR4 was associated with ROP16 on Chr. VIIb and VIR5 included an adenosine kinase on Chr. XII [Saeij *et al.*, 2006]. The strongest candidate for a virulence factor was ROP18 as it has been shown to be recruited to the surface of the PVM. Furthermore, analysis of gene expression showed that type III expressed considerable less ROP18 than types I or II. Sequencing of ROP18 revealed that type III had a 2.1-kilobase sequence inserted upstream of the start of the ROP18 gene. Introducing the ROP18 allele of type II ($ROP18_{II}$) into a type III background resulted in a 4-log increase of virulence compared to the wild-type type III [Saeij *et al.*, 2006]. When $ROP18_{I}$ was introduced into a type III background and inoculated into mice, a 4-fold increase in the number of parasites per vacuole was observed. Furthermore, virulence in mice increased 5-fold compared to type III. ROP 18 was further described as a pseudokinase. A point mutation introduced into $ROP18_{I}$ which disrupted its active site showed that the kinase activity is required for mediating enhanced virulence [Taylor *et al.*, 2006]. Comparing VIR4 between different types of *T. gondii*, which identified ROP16 on Chr. VIIb, it became apparent that $ROP16_{II}$ had 39 non-synonymous SNPs as compared to $ROP16_{I}$ and $ROP16_{III}$. Incorporation of $ROP16_{I}$ into a type II background (type II:$ROP16_{I}$) showed that ROP16 mediates

strain-specific activation of STAT3/6 and thus IL-12 secretion. In contrast to wild-type type II, type II:ROP_I induced STAT3 phosphorylation and thus inhibited IL-12 expression in infected HFF cells. The results provided a molecular basis for the strain-dependent differences in host cell responses to *T. gondii* infections. Type II, but not type I or III strains induced mouse macrophages to produce high levels of IL-12 to combat T. *gondii* infection [Saeij *et al.*, 2007]. All these studies were carried out in the light of the three major types existing in Europe and North America. However, descriptions of highly virulent *T. gondii* showing an atypical allele pattern by PCR-RFLP [Demar *et al.*, 2008; Dubey *et al.*, 2006a; Dubey *et al.*, 2008b; Pena *et al.*, 2008] and the first reports of clonal lineages from Brazil, which showed different virulence but were genetically distinct from types I, II and III, illustrated that further studies on the previously described virulence markers were needed. The newly discovered Brazilian *T. gondii* isolates of clonal group BrI displayed high virulence in mice, BrII showed intermediate virulence whereas *T. gondii* of group BrIII showed no virulence in mice. Importantly, many isolates displaying atypical alleles had low virulence in mice [Pena *et al.*, 2008]. Analysis of 25 *T. gondii* isolates representing 11 haplogroups were analysed for their ROP18 genetic diversity within eight introns from five unlinked loci. The results showed that, despite the high genetic diversity between haplogroups, only three alleles of ROP18 existed, named ROP18I*, ROP18II* and ROP18III* that corresponded to the different alleles previously described in types I, II and III. Nevertheless the genetic diversity in ROP18 was greater than in other *T. gondii* proteins such as SAG1, SAG2 or GRA3. Furthermore, ROP18 had a higher level of non-synonymous polymorphisms (pNS), i.e. these are base substitutions that might lead to missense or non-sense mutations, than surface antigens or house-keeping genes, which had mostly synonymous polymorphisms (pS). An elevated ratio of pNS/pS was detected in ROP18 suggesting a selective pressure on ROP18. Expression analysis between different isolates further showed an increased expression of ROP18 in isolates carrying ROP18I* as compared to ROP18III*. However, expression of ROP18 in *T. gondii* isolates with ROP18II* ranged from low to

high expression. Sequencing of ROP18 in different clonal types confirmed the presence of a 2.1 kb insert upstream of ROP18 (UPS-ROP18) in ROP18III* isolates. The presence or absence of UPS-ROP18 can be detected by conventional PCR. Furthermore, almost all avirulent *T. gondii* isolates analysed had the UPS-ROP18, whereas UPS-ROP18 was absent in virulent isolates carrying ROP18I*, but also in those with ROP18II*. Comparison of ROP18 in *T. gondii* with the closely related protozoan *N. caninum* revealed the existence of UPS-ROP18 similar to ROP18III* isolates [Khan *et al.*, 2009]. Both protozoans were shown to share a common ancestor [Su *et al.*, 2003] which led to the hypothesis that the UPS-ROP18 was lost by deletion or rearrangement in the more recent ancestors of ROP18I* and ROP18II*. Seventeen of 18 ROP18I* carrying isolates were virulent in mice, while 6/8 ROP18III* carrying *T. gondii* isolates were avirulent. Interestingly, high-virulent isolates carrying ROP18III* (CASTELLS and P89) showed no difference to the UPS-ROP18, and the coding sequence of ROP18 did not differ between isolates of the same group. Therefore, other genetic factors must confer virulence in these isolates [Khan *et al.*, 2009].

ROP18 was shown to promote the growth of *T. gondii* in inflammatory monocytes. While type I expressing ROP18 ($ROP18_I$) underwent rapid expansion in the peritoneum of infected mice, type III *T. gondii* remained at background levels. Further analysis showed that $ROP18_I$ parasites resided in intact vacuoles while ROP18III were associated with highly damaged vacuoles that underwent destruction. Introduction of ROP_I into a type III background showed that ROP18 was associated with the recruitment of IRGs. The level of ROP18 on vacuoles of ROP_I:type II parasites was inversely correlated with Irgb6 staining, which suggested that high level expression of ROP18 blocked recruitment of IRGs. Further experiments showed that ROP18 co-precipitated with Irgb6 and that active ROP18 was able to phosphorylate Irgb6 and Irgb10 in activated monocytes. Studies using small interfering RNAs (siRNA) to knock out Irgb6 resulted in the inhibition of ROP18-knockout parasite clearance thus reversing the effect shown in activated macrophages. It was shown that enhanced virulence of $ROP18_I$ in mice

was associated with the ability to phosphorylate IRGs and thus prevented parasite clearance by macrophages [Fentress *et al.*, 2010]. Another target of ROP18 was discovered to be the host endoplasmic reticulum-bound transcription factor ATF6β. ROP18 binds to the C-terminal of ATF6β and triggers degradation of the transcription factor [Yamamoto *et al.*, 2011].

Several researchers recently identified an additional virulence factor corresponding to VIR1 on Chr. XII. A ROP5 coding sequence was initially identified as the potential single locus conferring virulence but was later shown to be a cluster of tandemly repeated genes coding for this kinase. Type I has 6, type II 10 and type III has 4 tandem copies of the ROP5 pseudokinase gene. Also, all clonal types have three major isoforms of this pseudokinase, A, B and C. However, the ROP5 gene clusters of type I and type III were shown to be nearly identical and possess, in contrast to type II, virulent gene versions of ROP5. A frameshift in type II ROP5B seemed to result in a non-functional protein. However, the increased copy number appears to compensate for the defective version. ROP5-knockout parasites (ΔROP5) were used to discover which isoforms were needed to increase the virulence of *T. gondii* for mice. Insertion of a single copy of isoform $ROP5A_{III}$ into ΔROP5 restored virulence. It was further increased by inserting two copies of $ROP5A_{III}$ into ΔROP5 and insertion of a single copy of $ROP5A_{III}$ and $ROP5B_{III}$. However deletion of ROP5 had a more pronounced effect on virulence than deletion of ROP18. It has therefore been suggested that ROP5 acts independently of ROP18 and ROP16 [Behnke *et al.*, 2011; Ong *et al.*, 2010; Reese and Boothroyd, 2011; Reese *et al.*, 2011].

Other research on ROP16 indicated that its kinase activity is essential for STAT3 activation and that a single amino acid polymorphism determines the potency of the activation. This was proven by generating ROP16-deficient type I parasites which showed a marked reduction in STAT3 activation resulting in increased IL-12 production in infected macrophages. This defect was fully restored by complementation with $ROP16_I$ but not with $ROP16_{II}$. This work showed that

the ROP16 gene is responsible for STAT3 activation and thus for the suppression of *T. gondii*-induced inflammatory cytokines [Yamamoto *et al.*, 2011].

1.6 Aim of the study

To date, there is only limited data available concerning *T. gondii* genotypes in Germany. Preliminary studies indicated a high prevalence of type II in European countries including Germany. It has been shown, however, that other genotypes circulate in countries bordering Germany, such as Poland. No data existed on *T. gondii* genotypes in humans for Germany. To describe the genetic diversity of *T. gondii* in Germany, feline faecal samples were examined for the presence of *T. gondii* oocysts. Oocysts were isolated and genotyped using nine genetic markers. Isolated oocysts were bioassayed in mice and further propagated *in-vitro*. *In-vivo* experiments were conducted to examine the virulence of these *T. gondii* field isolates. To identify *T. gondii* genotypes in humans, vitreous fluid from patients with ocular toxoplasmosis was analysed for *T. gondii*-DNA and genotyped accordingly.

2 Materials and Methods

2.1 Animal models

IFN-γ knockout (GKO) mice (C.129S7(B6)-Ifngtm1Ts/J) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA), bred at the Friedrich-Loeffler-Institut and used to obtain *T. gondii* isolates from oocysts.

BALB/c mice (BALB/cAnNCrl) were purchased from Charles River (Sulzfeld, Germany), bred at the Friedrich-Loeffler-Institut and used for *in-vivo* virulence studies.

All mice were housed in standard cages and handled according to national guidelines. All animal experiments were approved by the Ministerium für Landwirtschaft, Umweltschutz und Raumordnung of the German Federal State of Brandenburg.

2.1.1 GKO mouse infection with sporulated oocysts

Prior to inoculation, oocysts kept in $K_2Cr_2O_7$ solution were washed four times by centrifugation (1,100 x g, 7 min, without brake) and resuspended in 15 ml tap water. The resulting pellet was finally resuspended in 1 ml tap water. Sporulated oocysts were counted using a Neubauer chamber. GKO mice were infected by oral gavage with 10^3 sporulated oocysts in a volume of 0.2 ml. When first clinical signs were detected, the mice were sacrificed and necropsied.

2.1.2 *T. gondii* virulence studies in BALB/c mice

Two to three months old BALB/c mice were used in all experiments. For intra-peritoneal infection, tachyzoites were grown and extracted from host cells by passage through a 27-gauge needle, washed twice in RPMI and quantified using a Neubauer chamber. Parasites were diluted in RPMI, and groups of five mice per dose inoculated intraperitoneally with 10^6, 10^4, 10^2 or 10 tachyzoites of each clone (in 500 µl) using a 27G needle. Each *in-vivo* experiment was carried out alongside a negative control group inoculated with RPMI only. Weight and mortality of the animals were daily recorded for 30 days after infection. Blood samples were taken at 0, 7, 14, 21, 28 and 30 d.p.i.. Mice were sacrificed at more than 20 per cent weight loss. All animals were necropsied and their organs weighted.

2.2 Molecular methods

2.2.1 DNA isolation

2.2.1.1 Oocyst-DNA

DNA was extracted from oocysts as described previously [Schares *et al.*, 2008b]. Briefly, oocysts stored in 1–2% $K_2Cr_2O_7$ were washed four times by centrifugation (1,100 x g, 7 min, without brake) in 15 ml PBS. The pellet, containing oocysts and remaining contaminants, was incubated in 2 ml 4% NaOCl (30 min, 37°C), then filled up to 12 ml with double-distilled H_2O and centrifuged (1,100 x g, 7 min, without brake). The pellet was resuspended and washed three times with PBS (1,100 x g, 7 min, without brake). After the last washing step, the pellet was transferred into a 1.5 ml reaction tube and subjected to three freeze (-20°C, 10 min) / thaw (room temperature, 2 min) cycles. It was then resuspended and incubated in 100 µl OOC-lysis buffer (600 mM EDTA, 1.3% (v/v) N-lauroylsarcosine, 2 mg/ml Proteinase K) for 45 min at 65°C. Thereafter, 400 µl OOC-CTAB buffer (2% (w/v) cetyl-trimethyl ammonium bromide, 1.4 M NaCl, 0.2% (v/v) mercaptoethanol, 20 mM EDTA, 100 mM Tris(hydroxymethyl)aminomethane were add-

ed and the suspension incubated for 60 min at 60°C. DNA was extracted with 500 µl phenol/chloroform/isoamylalcohol (in a 25:24:1 ratio) until the supernatant was without visible contaminants and collected into a fresh 1.5 ml reaction tube. After the addition of 0.04 vol of 4 M NaCl, the DNA was precipitated with 2 vol absolute ethanol, washed once with 70% (v/v) ethanol, dried and resuspended in 100 µl sterile double-distilled water. DNA was stored at 4–8°C until analysed. Aliquots of 1–2.5 µl were used for PCR.

2.2.1.2 Tachyzoite-DNA

DNA was extracted from pellets of *in-vitro* grown tachyzoites using a commercial kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) according to the manufacturer's instructions.

2.2.1.3 DNA from tissue samples

Tissue samples were collected and 100 mg weighed up for DNA isolation. DNA was isolated from the tissue samples using a standard protocol with some modifications [Strauss, 2001]. Briefly, tissue was digested overnight at 55°C in 600 µl lysis buffer (7.5 M ammonium acetate, 25 mM ethylene diamine tetra acetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS), 10 mM Tris pH 8.0 (Sigma, Germany), 0.2 mg/ml Proteinase K (Applichem, Germany)) shaking continuously in a thermo mixer (Eppendorf, Germany). DNA was extracted with 600 µl phenol/chloroform/isoamylalcohol (in a 25:24:1 ratio) until the supernatant was without visible contaminants and collected in a fresh 1.5 ml reaction tube. After the addition of 0.04 vol of 4 M NaCl, the DNA was precipitated with 2 vol absolute ethanol, washed once with 70% (v/v) ethanol, dried and resuspended in 100 µl sterile 0.1% TE-buffer. DNA was stored at 4–8°C until analysed.

2.2.2 Diagnostic PCR

For all PCR reactions, HPLC-purified primers were used at a final concentration of 0.5 mM, dNTPs at a final concentration of 250 mM each (Stratec Molecular, Germany) and Taq Polymerase (Stratec Molecular, Germany) at a final concentration of 1 U/25 µl with the buffer system supplied with the enzyme (Stratec Molecular, Germany). DNA extracted from oocysts and tachyzoites was first analysed by PCR using the primers listed in **Table 9**. PCR cycling conditions were as follows:

(1) COC-1/COC-2, JS4/5, Np6+/Np21+: 5 min at 94°C followed by 10 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C followed by 40 cycles of 1 min at 94°C, 1 min at 51°C, 1 min at 72°C and a final extension of 10 min at 72°C.

(2) TOX4/TOX5, TOX5/Tox-8: 1 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and a final extension of 10 min at 72°C.

(3) Hham34F/Hham3R: 1 min at 94°C followed by 35 cycles of 5 min at 94°C, 1 min at 60°C, 1 min at 72°C and a final extension of 10 min at 72°C.

Oocysts samples in which *T. gondii* was detected (i.e. amplification of the relevant PCR-product using TOX4/TOX5 and TOX5/Tox-8 primers) were identified and designated according to their origin. Designation was chosen as follows: TG-GERxxx for cats from Germany, where xxx corresponds to the isolate number. Cats from other countries were designated as TG-CCCxxx, where CCC stands for the 3-letter country code. *T. gondii* positive dog samples were designated TG-dgGERxxx or TG-dgCCCxxx.

Table 9: PCR primers used for diagnosis of DNA isolated from oocysts.
F: forward; R: reverse

Locus	Parasite	Primer	Sequence (5'- 3')	Reference
ITS-1	Coccidian	R: COC-1	AAGTATAAGCTTTTATACGG CT	[Ho *et al.*, 1996]
		R: COC-2	CACTGCCACGGTAGTCCAA TAC	
529bp repeat	*T. gondii*	F: TOX4	CGCTGCAGGGAGGAAGAC GAAAGTTG	[Homan *et al.*, 2000]
		R: TOX5	CGCTGCAGACACAGTGCAT CTGGAT	
529bp repeat	*T. gondii*	F: TOX5	CGCTGCAGACACAGTGCAT CTGGAT	[Homan *et al.*, 2000; Reischl *et al.*, 2003]
		R: Tox-8	CCCAGCTGCGTCTGTCGGG AT	
ITS-1	*N. caninum*	F: Np6+	CTCGCCAGTCAACCTACGT CTTCT	[Müller *et al.*, 1996]
		R: Np21+	CCCAGTGCGTCCAATCCTG TAAC	
ITS-1	*H. heydorni*	F: JS4	CGAAATGGGAAGTTTTGTG AAC	[Slapeta *et al.*, 2002]
		R: JS5	CAGCAGCTACATACGTAGA	
ITS-1	*H. hammondi*	F: Hham34F	ATCCCATTCCGGCTTCAGT	[Schares *et al.*, 2008a]
		R: Hham3R	CTTTCACAGCGGAGCCGAA GTTGGTTT	

2.2.3 Genotyping by PCR-RFLP

Genotyping was carried out using standard protocols as described [Herrmann *et al.*, 2010; Su *et al.*, 2006]. Loci of genetic marker regions were amplified by multiplex nested-PCR using the primers listed in **Table 10**. For all PCR reactions, HPLC-purified primers were used at a final concentration of 0.5 mM, dNTPs at a final concentration of 250 µM each (Stratec Molecular, Germany) and

Taq Polymerase (Stratec Molecular, Germany) at a final concentration of 1 U/25 µl with the buffer system supplied with the enzyme (Stratec Molecular, Germany). External PCRs were performed using the following cycling conditions: 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 55°C, 2 min at 72°C and a final extension of 10 min at 72°C. Internal PCRs for loci newSAG2, c22-8, c29-2, L358, PK1 and Apico were performed using the following cycling condition: 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 60°C, 2 min at 72°C and a final extension of 10 min at 72°C. Internal PCRs for loci BTUB and GRA6 were conducted as follows: 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final extension of 5 min at 72°C. Internal PCR for locus SAG3 was done as follows: 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 65°C, 1 min at 72°C and a final extension of 5 min at 72°C.

PCR-amplification of additional chromosomal marker regions was carried out using standard protocols as described [TGMD]. Loci of genetic marker regions were amplified by PCR using the primers listed in **Table 10**. For all PCR reactions, HPLC-purified primers were used at a final concentration of 0.5 mM, dNTPs at a final concentration of 250 µM each (Stratec Molecular, Germany) and Taq Polymerase (Stratec Molecular, Germany) at a final concentration of 1 U/25 µl with the buffer system supplied with the enzyme (Stratec Molecular, Germany). The PCRs were performed using the following cycling condition: 5 min at 94°C followed by 39 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final extension of 5 min at 72°C.

Table 10: PCR primers and restriction enzymes (RE) used for nested multilocus PCR-RFLP.

ext: external; int: internal; F: forward; R: reverse; min: minutes; h: hour; *digest conditions: restriction enzymes, buffer, temperature and time

Locus	Primers	Sequence (5'-3')	Digest*	Reference
Primers used to amplify loci for genotyping *T. gondii*				
newSAG2	SAG2-Fext:	GGAACGCGAACAATGAGTTT	TaqI + HinfI,	[Khan *et al.*,
	SAG2-Rext:	GCACTGTTGTCCAGGGTTTT	TaqI, 37°C for	2005a; Su *et*
	SAG2-Fint:	ACCCATCTGCGAAGAAAACG	30 min, 65°C for	*al.*, 2006]
	SAG2-Rint:	ATTTCGACCAGCGGGAGCAC	30 min	
SAG3	SAG3-Fext:	CAACTCTCACCATTCCACCC	BcnI, Tango,	[Grigg and
	SAG3-Rext:	GCGCGTTGTTAGACAAGACA	37°C for 1 h	Boothroyd,
	SAG3-Fint:	TCTTGTCGGGTGTTCACTCA		2001; Su *et*
	SAG3-Rint:	CACAAGGAGACCGAGAAGGA		*al.*, 2006]
BTUB	BTUB-Fext:	TCCAAAATGAGAGAAATCGT	TaqI+ Bsh1285l,	[Khan *et al.*,
	BTUB-Rext:	AAATTGAAATGACGGAAGAA	TaqI, 37°C for	2005a; Su *et*
	BTUB-Fint:	GAGGTCATCTCGGACGAACA	1 h, 65°C for 1 h	*al.*, 2006]
	BTUB-Rint:	TTGTAGGAACACCCGGACGC		
GRA6	GRA6-Fext:	ATTTGTGTTTCCGAGCAGGT	Tru1I, buffer R,	[Fazaeli *et*
	GRA6-Rext:	GCACCTTCGCTTGTGGTT	65°C for 1 h	*al.*, 2000; Su
	GRA6-Fint:	TTTCCGAGCAGGTGACCT		*et al.*, 2006]
	GRA6-Rint:	TCGCCGAAGAGTTGACATAG		
c22-8	c22-8-Fext:	TGATGCATCCATGCGTTTAT	Alw26I+MobII,	[Su *et al.*,
	c22-8-Rext:	CCTCCACTTCTTCGGTCTCA	Tango, 37°C for	2006]
	c22-8-Fint:	TCTCTCTACGTGGACGCC	30 min	
	c22-8-Rint:	AGGTGCTTGGATATTCGC		
c29-2	c29-2-Fext:	ACCCACTGAGCGAAAAGAAA	RsaI+TaiI, Tan-	[Su *et al.*,
	c29-2-Rext:	AGGGTCTCTTGCGCATACAT	go, 37°C for 1 h,	2006]
	c29-2-Fint:	AGTTCTGCAGAGTGTCGC	65°C for 1 h	
	c29-2-Rint:	TGTCTAGGAAAGAGGCGC		
L358	L358-Fext:	TCTCTCGACTTCGCCTCTTC	BsuRI+Hin1II,	[Su *et al.*,
	L358-Rext:	GCAATTTCCTCGAAGACAGG	buffer R, 37°C	2006]
	L358-Fint:	AGGAGGCGTAGCGCAAGT	for 1 h	
	L358-Rint:	CCCTCTGGCTGCAGTGCT		
PK1	PK1-Fext:	GAAAGCTGTCCACCCTGAAA	Eco99I+RsaI,	[Su *et al.*,
	PK1-Rext:	AGAAAGCTCCGTGCAGTGAT	Tango, 37°C for	2006]
	PK1-Fint:	CGCAAAGGGAGACAATCAGT	1 h	
	PK1-Rint:	TCATCGCTGAATCTCATTGC		

Apico	Apico-Fext:	TGGTTTTAACCCTAGATTGTGG	BspTI+HpyF3I,	[Su *et al.*,
	Apico-Rext:	AAACGGAATTAATGAGATTTGAA	Tango, 37°C for	2006]
	Apico-Fint:	TGCAAATTCTTGAATTCTCAGTT	1 h	
	Apico-Rint:	GGGATTCGAACCCTTGATA		
Primers used to amplify additional chromosomal loci				
AK16	AK16-F	TGCGTCTTTCTCAGAGTTG	HinfI+Lsp1109I,	[TGMD]
	AK16-R	CAGGAGAAAAGGTAACTGGA	buffer R, 37°C	
			for 1 h	
AK22	AK22-F	GGCAACAAGATCCAGTTCGT	BsuRI, buffer R,	[TGMD]
	AK22-R	CAGTTTTCTGCATCCACGAG	37°C for 1 h	
AK53	AK53-F	TTCGTCGCATACCCACAGTA	HpyF3I, Tango,	[TGMD]
	AK53-R	TCATCCCTAGAGCCTCCTCA	37°C for 1 h	
AK69	AK69-F	ACGAGCAACCATATCTTACC	HinfI, buffer R,	[TGMD]
	AK69-R	CGAACGGACAACAAGCTA	37°C for 1 h	
AK97	AK97-F	CTCCATCCTCTTTTGTTCCT	BseDI, Tango,	[TGMD]
	AK97-R	AAGACGGAAACACAAAATGT	55°C for 1 h	
L53	L53-F	CTCGTTGCGTTCCTTTGACT	BseRI, buffer R,	[TGMD]
	L53-R	GGAGAGAAGCTCGCAGTGTC	37°C for 1 h	
L375	L375-F	GAAGGTTCCGATCCATGTTG	MboI, buffer R,	[TGMD]
	L375-R	CGACCAAGCAACTGTCTCAA	37°C for 1 h	

2.2.4 Analysis using virulence markers and DNA sequencing

Virulence marker CS3 was analysed by using the standard PCR protocol as described for genotyping newSAG2 [Pena *et al.*, 2008] but using the primers listed in **Table 11**.

To detect the absence or presence of UPS-ROP18 [Khan *et al.*, 2009], the two primer pairs listed in **Table 11** were used in a conventional PCR with the following cycling conditions: 5 min at 95°C followed by 35 cycles of 30 s at 4°C, 30 s at 56°C and 2 min at 72°C.

For all PCR reactions HPLC-purified primers were used at a final concentration of 0.5 mM, dNTPs at a final concentration of 250 µM each (Stratec Molecular, Germany) and Taq Polymerase (Stratec Molecular, Germany) at a final concentration of 1 U/25 µl with the buffer system supplied with the enzyme (Stratec Molecular, Germany).

UPS-ROP18 was sequenced using the primers listed in **Table 11**. Primers to sequence UPS-ROP18 were designed using DNA sequences for type I (GT1), type II (ME49) and type III (VEG) deposited at toxodb.org. The full length sequence of type III ROP18 including the UPS was recovered by using the BLAST function and identified as TGVEG_chrVIIa1490900-1500951. Primers were designed using Lasergene 8.0 software. All primers were tested in a conventional PCR first. To sequence the UPS-ROP18 region, sequencing primers were generated for amplifying products of approximately 600 bp in length with at least 100 bp overlap between the respective sequences. Amplification of the UPS-ROP18 of type I/II *T. gondii* clones was performed using primers UPS-ROP18-1F and UPS-ROP18-4F. UPS-ROP18 type III *T. gondii* clones were sequenced after conventional PCR with unlabelled primers UPS-ROP18-1F/UPS-ROP18-2R and UPS-ROP18-3F/UPS-ROP18-4F. Direct sequencing of amplification products was carried out using a sequencing kit (Thermo Sequenase DYEnamic Direct Cycle, GE Healthcare, Germany) and the primers listed in **Table 11**. All forward primers were labelled with DY782, reverse primers with DY682 at their 5'end. Each DNA sample was sequenced in a LI-COR DNA Sequencer 4200 (MWG Biotech, Germany). Consensus sequences were assembled using the Lasergene 8.0 software (DNA Star Inc., Madison, USA) and compared with sequences of *T. gondii* type I (GT1), type II (ME49) and type III (VEG).

Table 11: PCR primers and restriction enzymes (RE) used for PCR-RFLP, conventional PCR and UPS-ROP18 sequencing.

ext: external; int: internal; F: forward; R: reverse; min: minutes; h: hour; *digest conditions: restriction enzymes, buffer, temperature and time

Locus	Primers	Sequence (5'-3')	Digest*	Reference
CS3	CS3-Fext:	GTGTATCTCCGAGGGGGTCT	Hin1II+MboI,	[Pena *et al.*,
	CS3-Rext:	TGTGACTTCTTCGCATCGAC	buffer G, 37°C	2008]
	CS3-Fint:	AGCGGATTTCCAACACTGTC	for 1 h	
	CS3-Rint:	CTGCTGCATTCACAAACTCC		
UPS-ROP18	Insert ROP18F	CACAGCATGAGCTTAAGAGTTG		[Khan *et al.*, 2009]
	Insert ROP18R	CACCGCAAGACAGGCTGTCTTC		
	AbsROP18F	CTAGCCACGCTATGCACCTCT		
	AbsROP18R	GCAAGTCACGCATAGTCTCATC		
UPS-ROP18 DNA-Sequencing	UPS-ROP18-1F	AACTGCCCGCCCTCTTTC		This study
	UPS-ROP18-1R	AACTTCCGGCTAACCTGACTGACA		This study
	UPS-ROP18-2F	AACGTGCATCTTCGGGTAACAAAC		This study
	UPS-ROP18-2R	GCGCTCGCCATCTTCTCAA		This study
	UPS-ROP18-3F	ACGCGGTTGTAGCTCCTCTGAAT		This study
	UPS-ROP18-3R	TTGTCTCGTTGGCTGCATCTTTGA		This study
	UPS-ROP18-4F	ATGTTACCGTCCTCCGTTTTTCTC		This study
	UPS-ROP18-4R	CACCGCAAGACAGGCTGTCTTC		[Khan *et al.*, 2009]

2.3 Parasitological methods

2.3.1 Faecal sample collection

Faecal samples from cats and dogs were provided by two veterinary laboratories: VetMed Labor GmbH (Ludwigsburg) and Veterinärmedizinisches Labor Freiburg (Freiburg). Both laboratories received feline and canine faecal samples collected by cat owners and veterinarians. Canine faecal samples from another project regarding *N. caninum* in dogs were also analysed for the presence of *T. gondii*. Samples were examined by a conventional floatation method using 262 mg/ml $ZnCl_2$ and 275 mg/ml NaCl [Schares *et al.*, 2005]. Floated material was

transferred onto a slide and examined by light microscopy using a magnification of at least 200x. When oocysts with a diameter of about 9–14 µm were detected, the remaining sample was posted to the Friedrich-Loeffler-Institut (FLI), Wusterhausen, Germany.

2.3.2 Oocyst isolation from faeces

Samples received at the FLI were examined by a combined sedimentation and floatation procedure as previously described [Schares *et al.*, 2005]. Routinely, 100 ml beakers and small sieves (diameter 7.5 cm diameter) were used for sedimentation. Faecal matter was mixed and put through a strainer into the conical beaker. Water was added up to the 100 ml mark and stirred to ensure that no material stack to the edges and wall of the beaker. Material was left to sediment for 1 hour (day 1). After sedimentation, the supernatant was poured into another conical beaker and left for sedimentation overnight (day 2). Concentrated sucrose solution was added to the sediment and transferred into a 50 ml tube followed by a centrifugation step of 10 min at 1,600 x g. To identify oocysts by light microscopy, a sample of the floating oocysts was taken and transferred onto a microscope slide. The oocyst sample was analysed and photographed using a 400x magnification for reference and identification purposes. Diameters of oocysts were measured. After centrifugation, 2 ml tap water was pipetted on top of the floatation fluid. The water phase was then carefully stirred with the use of a pipette tip. Between 3 and 4 ml of the upper phase containing oocysts were recovered. Resulting material was then washed 3 times in 50 ml tap water at 1,600 x g, 10 min without brake. The number of oocysts was estimated by microscopic examination of 10 µl using a Neubauer chamber and an aliquot of 10^4 oocysts was used for DNA isolation. If the concentration of oocysts was too low to count them in the Neubauer chamber, a 2.5 ml aliquot was used for DNA isolation. If 2.5 ml of oocyst suspension failed to yield sufficient DNA to amplify the target by PCR, a 5 ml aliquot was used for DNA isolation. In addition, all samples were examined for oocysts by light mi-

croscopy. Oocysts were allowed to sporulate by incubation at room temperature for 72 or 96 h under aerated conditions and then stored in $K_2Cr_2O_7$ at a final concentration of 1–2% (w/v) at 4–8°C until use.

2.3.3 Isolation of sporozoites from *T. gondii* oocysts (excystation)

A minimum of 10^6 *T. gondii* oocysts stored in 1–2% $K_2Cr_2O_7$ were washed four times in a 15 ml falcon tube by centrifugation (1,100 x g, 7 min, without brake) in 15 ml PBS. The pellet, containing oocysts and remaining contaminants, was incubated in 2 ml 4% NaOCl (15 min at 37°C), then filled up to 12 ml with double-distilled H_2O and centrifuged (1,100 x g, 7 min, without brake). The pellet was resuspended and washed three times with sterile Dulbecco's Minimum Eagle's Medium (DMEM) at 1,100 x g, 7 min, without brake. The supernatant was removed and 5 ml sterile DMEM added to the pellet. The ultrasound rod was washed three times with sterile DMEM before use. The suspension was treated with ultrasound four times for 30 s (50% active cycle, output level 3) with a 30 s break between cycles. The samples were kept on ice during the entire sonication procedure. The resulting solution was centrifuged at 1,100 x g for 7 min, without brake. The supernatant was discarded and the pellet resuspended in 2 ml 0.75% tauchocholat for 2 hrs at 37°C. The sample was washed with sterile DMEM and centrifuged at 1,100 x g for 7 min, without brake. The sample was checked for free sporozoites under the microscope (400x magnification). When free sporozoites were observed, the solution was added to a VERO cell monolayer. The cell culture was checked three times daily for plaque formation.

2.3.4 Isolation of tachyzoites from *T. gondii* by bioassay

Infected GKO and BALB/c mice were observed daily for any clinical signs. When the first clinical signs were observed, the infected mice were anaesthetised by injection of 0.1 ml of a xylazin/ketamin hydrochloride mixture [Schares *et al.*, 2008b] and sacrificed by blood withdrawal (heart puncture). Animals were

skinned and peritoneal fluid was recovered by washing the peritoneal cavity with 1 ml sterile DMEM. The recovered fluid was inspected for the presence of *T. gondii* tachyzoites under a microscope. If no tachyzoites were detected, half of the heart and brain (approximately 0.05 g and 0.2 g, respectively) were homogenised by pestle and mortar and 1 ml sterile RMPI medium was added. The recovered parasite suspension or the homogenized tissues (approximately 1 ml) were added to VERO cells. After 4 h cell culture medium was replaced with fresh medium. Cell cultures were checked for parasite growth daily.

2.3.5 *In-vitro* parasite maintenance

All *T. gondii* isolates derived from bioassay and *T. gondii* reference strains (RH, ME49 and NED) were maintained in VERO cell monolayers using Dulbecco's MEM with 2% foetal calf serum and 1% antibiotic solution (10,000 IU Penicillin and 10,000 µg Streptomycin/ml solution) at 37°C and 5% CO_2.

2.3.6 Tachyzoite isolation from cell culture for DNA or protein extraction

At least one day prior to harvesting cells, FCS-containing medium was replaced with FCS-free medium. Any remaining cells containing tachyzoites were scraped off using a cell scraper. All material was transferred to a 50 ml falcon tube and centrifuged for 10 minutes at 2,500 rpm, 4°C without brake. The resulting suspension was pressed through a syringe with a 27G needle and further filtered using a 5 µm sterile syringe filter (Sartorius, Germany). The total parasite number was calculated using the improved Neubauer chamber method.

2.3.7 Tachyzoite isolation from cell culture for *in-vivo* infections

To isolate parasites for *in-vivo* use, several VERO cell cultures were infected simultaneously to ensure that enough parasites could be harvested. Cells were checked daily to ensure the optimal harvesting time. At least two days prior to

harvesting, cells were kept in FCS-free medium. Parasites were harvested by transferring free tachyzoites into 50 ml falcon tubes. Tachyzoites were washed three times for 10 minutes in sterile PBS at 2,500 rpm, 4°C, without brakes. The concentration of live parasite was determined using the improved Neubauer chamber method in the presence of Trypan-Blue. The required number of live tachyzoites was prepared in DMEM medium and kept on ice until use.

2.3.8 Cloning of *T. gondii* by limiting dilution

Limiting dilutions were carried out in 96-well-plates (Greiner, Germany). VERO cells were seeded at 10^6 cells per well. Resulting monolayers were infected by adding 200 µl medium containing a calculated number of 0.1–0.5 parasites per well. When the first single plaque was observed, parasites were transferred into 25 cm^2 tissue culture flasks (Greiner, Germany) and clonally propagated in VERO cells as previously outlined.

2.4 Immunoblot analysis

TgSAG1 was obtained by immune affinity chromatography using the mouse monoclonal antibody (mAb) IgG2a P30/3 (ISL, UK) as described by [Maksimov *et al.*, 2011]. The purity of TgSAG1 was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and India ink staining [Schares *et al.*, 2000]. The amount of recovered TgSAG1 was determined relative to a standard of bovine serum albumin by silver staining [Heukeshoven and Dernick, 1988]. Affinity purification of TgSAG1 resulted in an antigen preparation that was free off detectable contaminating components [Hosseininejad *et al.*, 2009].

To detect antibody reactions against the immunodominant surface antigen TgSAG1 of *T. gondii*, previously described protocols were employed (Maksimov et al., 2011). SDS-PAGE and blotting were performed as described previously

[Maksimov *et al.*, 2011]. PVDF membranes with transferred TgSAG1 were cut into strips and blocked using PBS-TG (PBS-T, 2% (v/v) fish gelatine liquid (Serva, Germany) for 15 min. PBS-TG was removed and each strip was incubated with body or tissue fluid diluted 1:2 in PBS-TG for 1 h. Strips were washed five times using PBS-T followed by incubation with AffiniPure Rabbit Anti-Dog IgG+IgM (H+L) (Jackson ImmunoResearch, USA) diluted 1:250 in PBS-T for 1 h. Strips were then washed three times using PBS-T and two times using PBS and incubated with a substrate solution (40 µl H_2O_2 (30% (v/v)) and 30 mg 4-chloro-1-naphthol (Sigma–Aldrich, USA) in 40 ml PBS, 20% (v/v) methanol) for 20 min. Test results were regarded as positive if the TgSAG1 band at 30 kDa was detected.

2.5 Statistical methods

2.5.1 Analysis of feline faecal samples June 2007–December 2008

The 95% confidence intervals (CI) for prevalence data were calculated by approximation assuming a binomial distribution and an infinite cat population. Differences among the quarters of a year regarding the proportion of *T. gondii*-positive samples received by VetMed Labor GmbH in the different seasons were examined with the Fisher's Exact Test. To determine whether positive samples were overrepresented relative to negative samples in a particular age group, data were analysed by the Fisher's Exact Test. The spatial analysis of sample data was based on postcodes and samples without location data were excluded from the analysis. Prior to analysis, the data sets based on the postcode areas, were attributed to the administrative level of districts ('Landkreise' and 'kreisfreie Städte') for Germany (Bundesamt für Kartographie und Geodäsie, Frankfurt am Main, Germany). Population density data were available at the district level for the year 2007. Geographic analyses were performed using ArcGIS software version 9.3 (ESRI, Redlands, California, USA). The Mann-Whitney U test was used to com-

pare the population densities of districts where samples from *T. gondii*-positive and -negative cats had been submitted from (Statistica 7.1, Stat Soft Inc., Tulsa, USA). P values lower than 0.05 were considered statistically significant.

2.5.2 Analysis of virulence data

Weight data for each day and animal were recorded in Microsoft Excel spread sheets. Maximum weight loss was calculated using the Min() function of Excel and subsequently transferred to GraphPad Prism 4 Software (GraphPad Software, San Diego California USA) for further analysis. Using the statistical package included in Prism 4, each infection group at each dose was compared to the control animals using the Mann-Whitney U test. P values lower than 0.05 were considered statistically significant. Survival curves were created with GraphPad Prism4.

3 Results

3.1 Isolation and characterisation of animal samples

3.1.1 Isolation of *T. gondii* oocysts from faecal samples

Between June 2007 and December 2010, 258 feline and canine faecal samples from Germany containing *T. gondii*-like oocysts were received for further analysis. The two collaborating veterinary laboratories, VetMed Labor GmbH (Ludwigsburg) and Tierärztliches Labor Freiburg (Freiburg), submitted only faecal samples in which they had detected *T. gondii*-like oocysts with an average diameter of 9–14 µm. A total of 143 feline faecal samples and 115 canine faecal samples from Germany were analysed. Oocysts were isolated by sedimentation/floatation. DNA was isolated and species-specific PCRs for *T. gondii*, *H. hammondi*, *H. heydorni* and *N. caninum* carried out. An example of a diagnostic analysis is shown in **Figure 3**.

In 74/143 (51.75%) feline faecal samples and 5/115 (6.09%) of the canine faecal samples *T. gondii* oocysts were present as detected by sedimentation/flotation and subsequent specific PCR (**Figure 4**). 47/143 (32.87%) of the feline faecal samples and 5/115 (4.35%) of the canine faecal samples contained oocysts of *H. hammondi* (**Figure 5**). More than half of all analysed canine faecal samples contained oocysts of *H. heydorni* (58/115; 50.43%), whereas 10/115 (8.70%) of the canine faecal samples contained oocysts of *N. caninum*. None of the feline faecal samples contained *H. heydorni* or *N. caninum* oocysts. In two canine faecal samples (2/115; 1.74%), DNA of *H. heydorni* and of *N. caninum* was detected by specific PCR. It was concluded that a mixed infection had occurred in both cases. Also, 6/143 (4.20%) of the feline faecal samples and 13/115 (11.30%) of the canine faecal samples were PCR-positive for coccidian-specific PCR, but PCR-negative for *T. gondii*, *H. hammondi*, *H. heydorni* and *N. caninum*.

These cats and dogs were possibly infected with a coccidian parasite different from the tested species. They were therefore further analysed for the presence of *Besnoitia besnoiti* DNA. None of the coccidian positive faecal samples from Germany tested PCR-positive for *B. besnoiti* by PCR. A small number of feline faecal sample (15/143; 10.49%) and of canine faecal samples (19/115; 16.52%) containing at least one oocysts, as analysed by light microscopy after sedimentation/floatation at the veterinary laboratories, tested PCR-negative for coccidian specific DNA at the FLI. The parasites in these samples remained unidentified. In cases where the PCR-specific signals for *T. gondii* were weak, the bioassay was carried out to isolate *T. gondii*. A total of 29 feline and 35 canine faecal samples from outside Germany were also analysed. Two of 35 (5.71%) canine faecal samples and 11 of 29 (37.93%) feline faecal samples tested *T. gondii* PCR-positive.

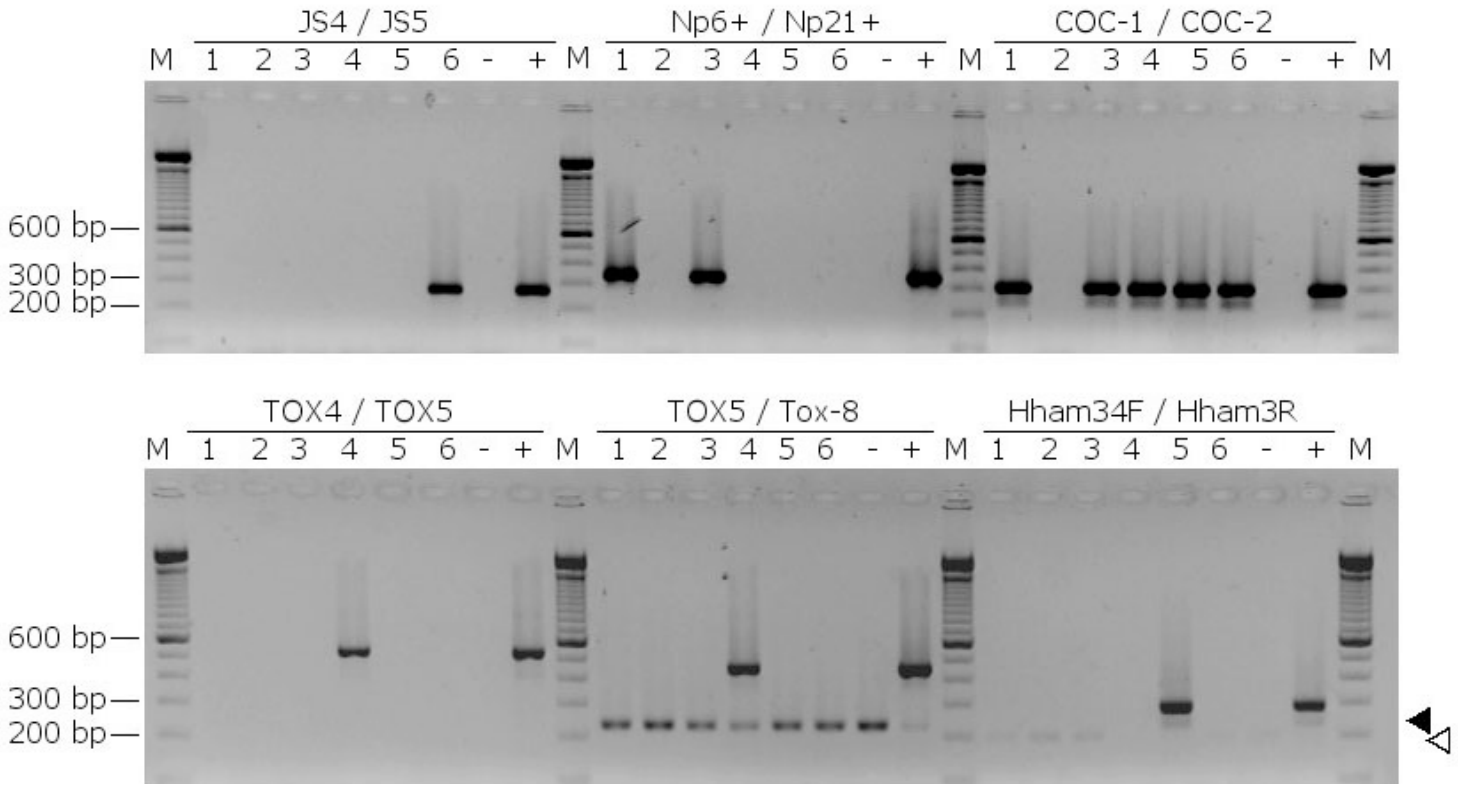

Figure 3: Analysis of oocyst DNAs isolated from faecal samples.

PCR-primer pairs specific for *H. heydorni* (JS4/5), *N. caninum* (Np6+/Np21+), coccidian (COC-1/COC-2), *T. gondii* (TOX4/TOX5 and TOX5/Tox-8) and *H. hammondi* (Hham34F/Hham3R) were utilised. Samples are PCR-positive for *N. caninum*, coccidian PCR-negative, *N. caninum*, *T. gondii*, *H. hammondi* and *H. heydorni*, respectively. M: molecular markers; 1-6: DNA samples from faecal samples, Triangles: internal PCR amplification control.

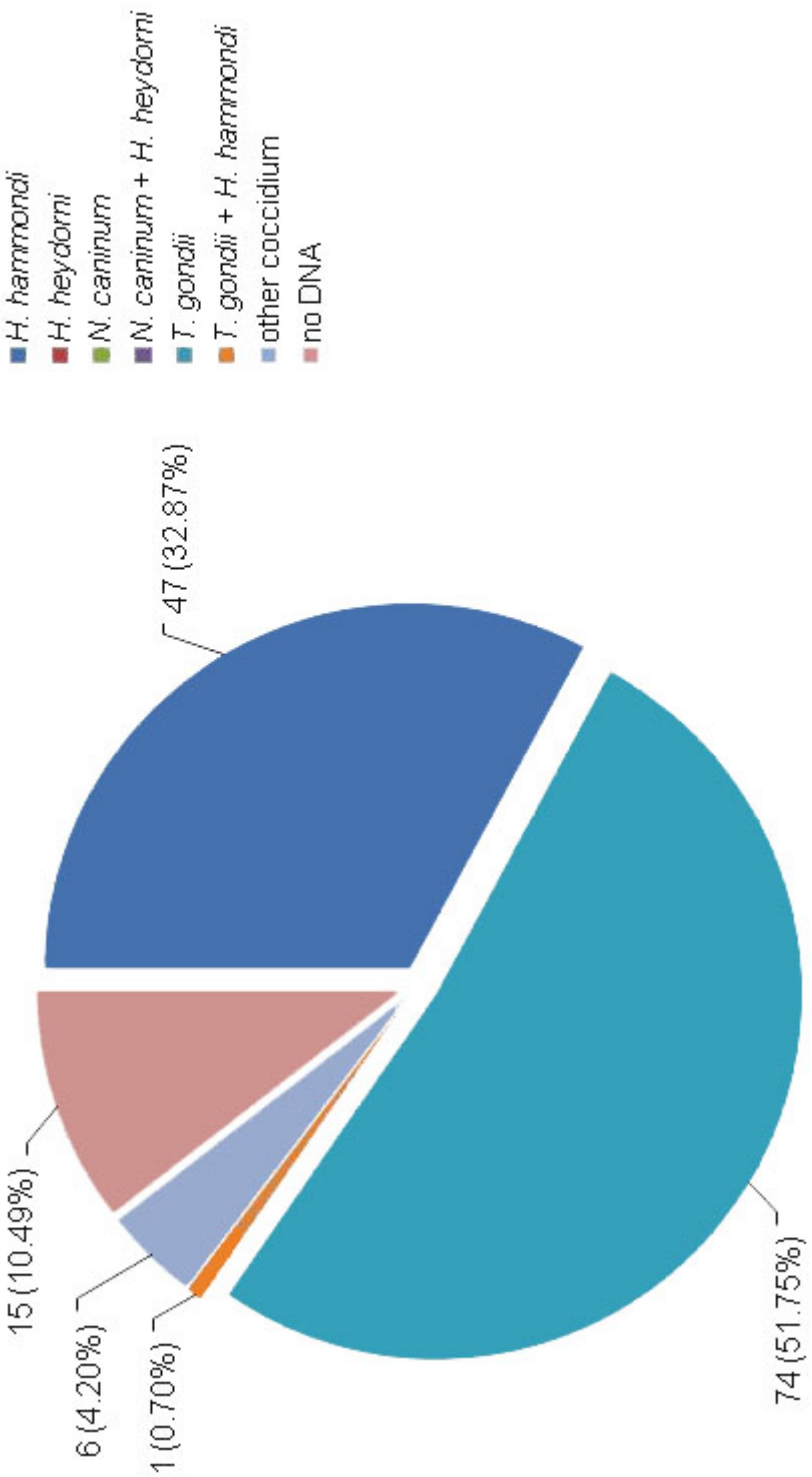

Figure 4: Proportion of feline faecal samples from Germany containing *T. gondii*-like oocysts. Samples were obtained between June 2007 and December 2010 and tested PCR-positive for listed coccidian parasites.

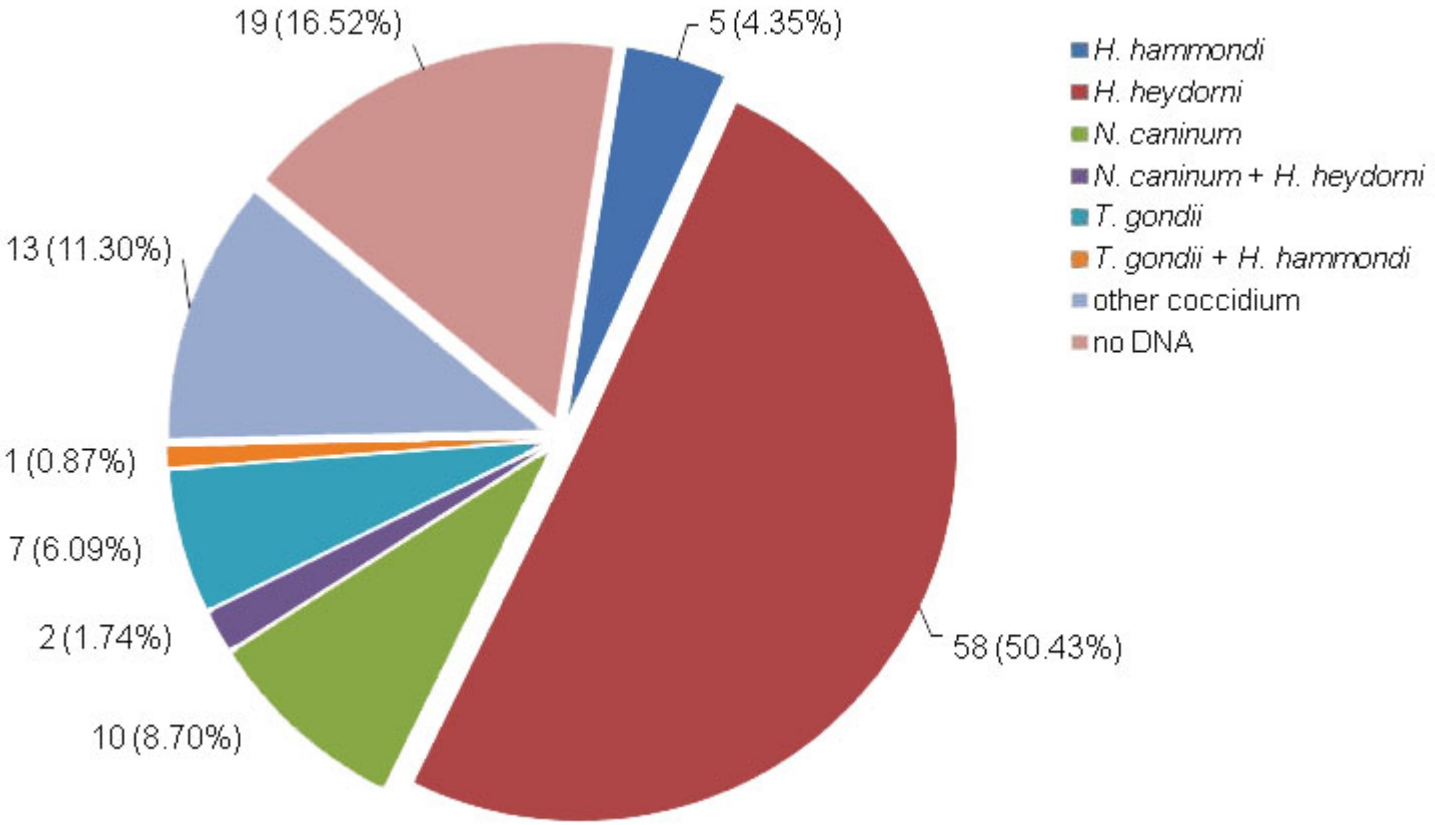

Figure 5: Proportion of canine faecal samples from Germany containing *T. gondii*-like oocysts.

Samples were obtained between June 2007 and December 2010 and tested PCR-positive for listed coccidian parasites.

3.1.2 Bioassay of *T. gondii* oocysts in GKO mice

The bioassay for *T. gondii* in GKO mice was carried out with oocyst samples that tested weakly *T. gondii* PCR-positive or contained little oocyst DNA. This way enough DNA might be generated by subsequent cell culture as high amounts of DNA are required for genotyping by PCR-RFLP. Furthermore, the objective was also to generate as many field isolates of *T. gondii* from Germany as possible for future reference and scientific research with regards to phylogeny and virulence studies. In cases where sporulated *T. gondii* (**Figure 6**) were observed and especially in cases where the PCR-specific bands for *T. gondii* were weak, bioassay in mice was carried out. Oocyst samples, in which *T. gondii* was detected by PCR, were identified and designated accordingly. In total, *T. gondii* tachyzoites could be isolated and further propagated in VERO cell culture from one *T. gondii* oocyst sample of canine origin (**Table 12**) and 33 *T. gondii* oocyst samples of feline origin (**Table 13**). One sample (TG-GER045) was used to liberate sporozoites from the oocysts and to isolate *T. gondii* tachyzoites in VERO cells without an initial passage through GKO mice. This was only possible due to the high number of sporulated oocysts (> 10^6 oocysts/ml) contained in the sample.

GKO mice inoculated with 10^3 sporulated oocysts usually showed clinical signs of infection, such as immobility, raised fur hair, hump-back and shaking head at 10 d.p.i. and were sacrificed when these signs were observed. Notable exceptions to his rules were infections with oocysts of sample TG-GER054 and TG-GER63. Only 10^2 oocysts were used to bioassay TG-GER054 thus explaining the late signs of disease (18 d.p.i.). As will be discussed later, TG-GER63 contained many highly mouse-virulent *T. gondii* clones that may have accelerated disease progression. Isolate TG-GER63 was also isolated from BALB/c mice (B136/1-5) following the isolation of *T. gondii* tachyzoites from GKO mouse K119/1. This was necessary to identify various *T. gondii* clones with differences in mouse virulence contained within the single oocysts sample TG-GER63. Clinical

signs of disease in BALB/c mice infected with *T. gondii* isolated from GKO mouse K119/1 were noted 6 days later than in GKO mice at 16 d.p.i.. Bioassay of *T. gondii* from mice failed for eight samples (Tg-dgGER08, TG-GER34, 46, 51, 55, 62, 85, 87). In these cases it seemed that the visible oocysts were either not yet fully sporulated or not infectious any longer.

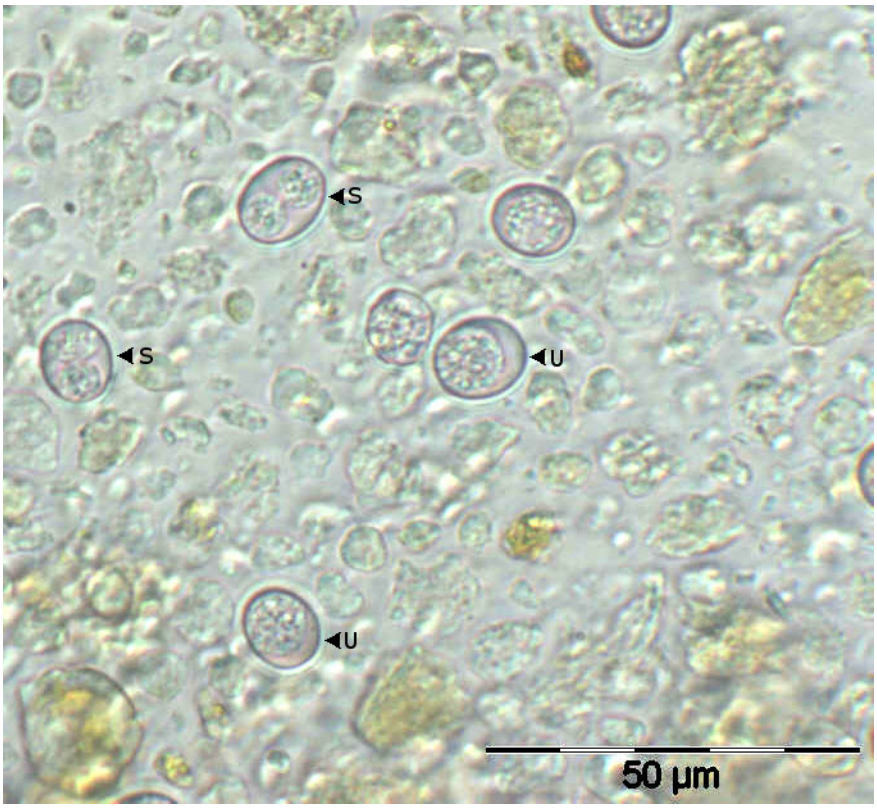

Figure 6: Microscopic examination of a feline faecal sample.

Sample shown contained sporulated (S) and unsporulated (U) *T. gondii* oocysts (400x magnification).

Table 12: Bioassay of *T. gondii* oocysts from canine faecal samples in mice.

Toxo-ID	Origin	Parasite stage used	ID	T_{death} (d.p.i.)	Mouse model
TG-dgGER05	Dog	Oocysts	K106	11	GKO
TG-dgGER08	Dog	Oocysts	K161	-	GKO

Table 13: Bioassay of *T. gondii* oocysts from feline faecal samples in mice.

Toxo-ID	Origin	Parasite stage	ID	T_{death} (d.p.i.)	Model
TG-GER24	Cat	Oocyst	K105	10	GKO-mouse
TG-GER28	Cat	Oocyst	K66	10	GKO-mouse
TG-GER30	Cat	Oocyst	K102	13	GKO-mouse
TG-GER32	Cat	Oocyst	K68	10	GKO-mouse
TG-GER33	Cat	Oocyst	K67	10	GKO-mouse
TG-GER34	Cat	Oocyst	K103	-	GKO-mouse
TG-GER35	Cat	Oocyst	K69	10	GKO-mouse
TG-GER36	Cat	Oocyst	K70	10	GKO-mouse
TG-GER36	Cat	Oocyst	K72	10	GKO-mouse
TG-GER37	Cat	Oocyst	K73	10	GKO-mouse
TG-GER38	Cat	Oocyst	K76	10	GKO-mouse
TG-GER39	Cat	Oocyst	K78	16	GKO-mouse
TG-GER40	Cat	Oocyst	K79	11	GKO-mouse
TG-GER41	Cat	Oocyst	K80	10	GKO-mouse
TG-GER42	Cat	Oocyst	K84	12	GKO-mouse
TG-GER43	Cat	Oocyst	K83	9	GKO-mouse
TG-GER45	Cat	Sporozoite	-	-	VERO cell
TG-GER46	Cat	Oocyst	K101	-	GKO-mouse
TG-GER49	Cat	Oocyst	K104	10	GKO-mouse
TG-GER50	Cat	Oocyst	K107	9	GKO-mouse
TG-GER51	Cat	Oocyst	K81	-	GKO-mouse
TG-GER54	Cat	Oocyst	K114	18	GKO-mouse
TG-GER55	Cat	Oocyst	K115	-	GKO-mouse
TG-GER61	Cat	Oocyst	K126	13	GKO-mouse
TG-GER62	Cat	Oocyst	K127	-	GKO-mouse
TG-GER63	Cat	Oocyst	K119/1	8	GKO-mouse
TG-GER63	Cat	Oocyst	K119/1	8	GKO-mouse
TG-GER63	Cat	Tachyzoite	B136/1	20	BALB/c-mouse
TG-GER63	Cat	Tachyzoite	B136/2	22	BALB/c-mouse
TG-GER63	Cat	Tachyzoite	B136/3	23	BALB/c-mouse
TG-GER63	Cat	Tachyzoite	B136/4	21	BALB/c-mouse
TG-GER63	Cat	Tachyzoite	B136/5	21	BALB/c-mouse
TG-GER63	Cat	Oocyst	K128/1	8	GKO-mouse
TG-GER63	Cat	Oocyst	K128/2	8	GKO-mouse
TG-GER63	Cat	Oocyst	K128/3	8	GKO-mouse
TG-GER63	Cat	Oocyst	K128/4	8	GKO-mouse
TG-GER78	Cat	Oocyst	K159	12	GKO-mouse
TG-GER79	Cat	Oocyst	K160	10	GKO-mouse
TG-GER85	Cat	Oocyst	K163	-	GKO-mouse
TG-GER87	Cat	Oocyst	K162	-	GKO-mouse

3.1.3 Seasonality, age and spatial distribution of cats shedding *T. gondii* oocysts

Statistical analysis of the age of oocyst-shedding cats and the seasonality of oocyst-shedding was only carried out for the period between 2007 and 2008 and for feline faecal samples submitted by VetMed Labor GmbH since for that period and from this particular laboratory all data sets were obtained for complete analysis. For each month, the total number of feline faecal samples received by VetMed Labor GmbH was inquired and correlated to the number of *T. gondii* positive faecal samples as analysed by PCR. The numbers of *T. gondii*-positive and -negative faecal samples were analysed by the Fisher's Exact Test for each month and summarised for each quarter (QT).

A total of 18,259 feline faecal samples were received for diagnosis at VetMed Labor GmbH. During the whole period, 45/18,259 (0.25%) cats shed *T. gondii* oocysts, whereas 33/18,259 (0.18%) cats shed *H. hammondi* oocysts. Among all the samples received at VetMed Labor GmbH between 2007 and 2008, the proportion of *T. gondii*-positive samples (n = 45; 0.25%) differed depending on the submission month and the quarter of the year (**Table 14** and **Table 15**). In the first (January–March), second (April–June), third (July–September) and fourth quarter (October–December), 0.16% (4/2,549), 0.03% (1/2,953), 0.37% (21/5,734) or 0.27% (19/7,023) of the samples tested positive for *T. gondii* (**Table 15**). To compare *T. gondii*-positive and -negative feline faecal samples quarter wise, Fisher's Exact Test was applied. The differences were only statistically significant (Fisher's Exact Test; $P > 0.05$) when the 2nd and the 3rd quarter (P = 0.002) and the 2nd and 4th quarter (P = 0.01) were compared (**Table 16**). When combining several months, statistically significant differences were observed between the 2nd quarter and quarters 1+3+4 as well as between the 3rd quarter and quarters 1+2+4. Following this analysis, the year was divided into two halves ranging from January-June and from July-December. *T. gondii* positive and negative faecal sample were again analysed for any statistically significant differences.

When the 1st half of the year (January–June) was compared to the 2nd half (July–December), a significantly higher proportion of samples tested positive for *T. gondii* oocysts in the 2nd half of the year ($P = 0.005$).

Table 14: Total number of samples analysed by VetMed Labor GmbH and number of samples analysed positive for *T. gondii* or *H. hammondi* per month.

Total number of samples and number of samples tested.

Month	Samples	*T. gondii* positive (%)	*H. hammondi* positive (%)
January	1,007	1 (0.10)	4 (0.40)
February	829	1 (0.12)	0 (0.00)
March	713	2 (0.28)	1 (0.14)
April	794	0 (0.00)	0 (0.00)
May	688	0 (0.00)	0 (0.00)
June	1,471	1 (0.07)	1 (0.07)
July	1,791	8 (0.45)	1 (0.06)
August	1,841	10 (0.54)	3 (0.16)
September	2,102	3 (0.14)	1 (0.05)
October	2,541	7 (0.28)	8 (0.31)
November	2,488	8 (0.32)	9 (0.36)
December	1,994	4 (0.20)	5 (0.25)
Totals	**18,259**	**45 (0.25)**	**33 (0.18)**

Table 15: Total number of samples analysed by VetMed Labor GmbH and number of samples tested positive for *T. gondii* or *H. hammondi* per quarter of the year.

Total number of samples and number of samples tested.

Quarter (QT), (period)	**Samples**	***T. gondii* positive (%)**	***H. hammondi* positive (%)**
QT1 (January–March)	2,549	4 (0.16)	5 (0.20)
QT2 (April–June)	2,953	1 (0.03)	1 (0.03)
QT3 (July–September)	5,734	21 (0.37)	5 (0.09)
QT4 (October–December)	7,023	19 (0.27)	22 (0.31)
Totals	**18,259**	**45 (0.25)**	**33 (0.18)**

Table 16: Statistical analysis (Fisher's Exact Test) of *T. gondii* oocyst shedding for different quarters of the year.

QT=Quarter, Statistically significant differences ($P < 0.05$, Fisher's Exact test) are indicated by bold letters.

Comparison	*T. gondii* positive; *T. gondii* negative [n]	*T. gondii* positive; *T. gondii* negative [n]	Significance [*P* =]
QT1 vs QT2	4; 2,545	1; 2,952	0.1893
QT1 vs QT3	4; 2,545	21; 5,713	0.1304
QT1 vs QT4	4; 2,545	19; 7,004	0.4780
QT2 vs QT3	1; 2,952	21; 5,713	**0.0024**
QT2 vs QT4	1; 2,952	19; 7,004	**0.0128**
QT3 vs QT4	21; 5,713	19; 7,004	0.3443
QT1 vs QT2+3+4	4; 2,545	41; 15,669	0.3959
QT2 vs QT1+3+4	1; 2,952	44; 15,262	**0.0070**
QT3 vs QT1+2+4	21; 5,713	24; 12,501	**0.0355**
QT4 vs QT1+2+3	19; 7,004	26; 11,210	0.6464
QT1+2 vs QT3+4	5; 5,497	40; 12,717	**0.0050**

Another aim of the study was to determine if *T. gondii* oocyst shedding could be correlated to a certain age or age group. The age ranges of the cats from which *T. gondii*-positive samples were obtained were therefore compared accordingly. Three categories of age ranges were formed: group 1) one year and younger, group 2) 1–7 years and group 3) older than seven years. Complete data sets were available for 34 cats. Among these animals, 18/34 (52.94%) positive cats were up to one year old. Sixteen animals (16/34; 47.06%) were older than one year of age. 6/34 (17.65%) of *T. gondii*-positive cats were between one and seven years old. Only five *T. gondii*-positive cats (5/34; 14.71%) were below the age of six months. Ten (10/34; 29.41%) cats were older than seven years and the oldest cat shown to shed *T. gondii* oocysts was 18 years old. The results are summarised in **Figure 7**. Samples from ten *T. gondii*-negative cats were randomly selected per month (i.e. a total number of 190 cats) and included into the statistical analysis. These samples allowed comparison of *T. gondii*-positive cats with *T. gondii*-negative cats with regards to (i) the age when they shed oocysts and (ii) the geographical locations where the cat samples had been submitted from. The age distribution among the *T. gondii*-positive cats was similar to that of the *T. gondii*-negative cats. In the negative sample 81/190 (42.63%) of the cats were up to one year old, 47/190 (24.74%) cats were 1–7 years old and 62/190 (32.63%) cats were older than seven years old. No statistically significant difference regarding the proportion of cats between *T. gondii*-positive and *T. gondii*-negative animals was observed (Fisher's Exact test; $P > 0.05$).

It was assumed that cats kept in rural areas were more susceptible to infection with *T. gondii* and thus more likely to shed oocysts. Cats in those areas were also more likely to roam freely and had thus easier access to infected intermediate host species of *T. gondii*, such as birds and rodents. It was also more likely that cats in such areas were fed less processed and tinned cat food thus increasing the likelihood of getting infected and to shed oocysts. It was thus hypothesised that cats originating from areas with a high population density were less likely to shed

oocysts than cats from rural areas with a lower population density. To test this hypothesis, the possible influence of human population density on the shedding of *T. gondii* oocysts of cats was also analysed. If the postal code of the place where the cat sample had been submitted from was available, the respective data set was analysed by comparing the geographical origin of the *T. gondii*-negative samples with that of the *T. gondii*-positive samples with regard to human population density at the district level. *T. gondii*-negative samples came from districts with a mean human population density of 710.50 (CI: 643.10–777.90) persons per square kilometre (km^2), whereas *T. gondii*-positive samples came from districts with a mean population density of 815.80 (CI: 666.20–965.20) persons per km^2. The differences in the population density were not statistically significant between the districts where negative and positive samples had been submitted from ($P > 0.05$; Mann-Whitney U Test). Only three out of 44 (6.82%) *T. gondii*-positive samples came from districts with a population density of less than 100 persons per km^2. Twelve positive samples (27.27%) originated from districts with a population density of 100–500 persons per km^2, whereas 29 positive samples (65.91%) came from districts with a population density of more than 500 persons per km^2 (**Figure 8**).

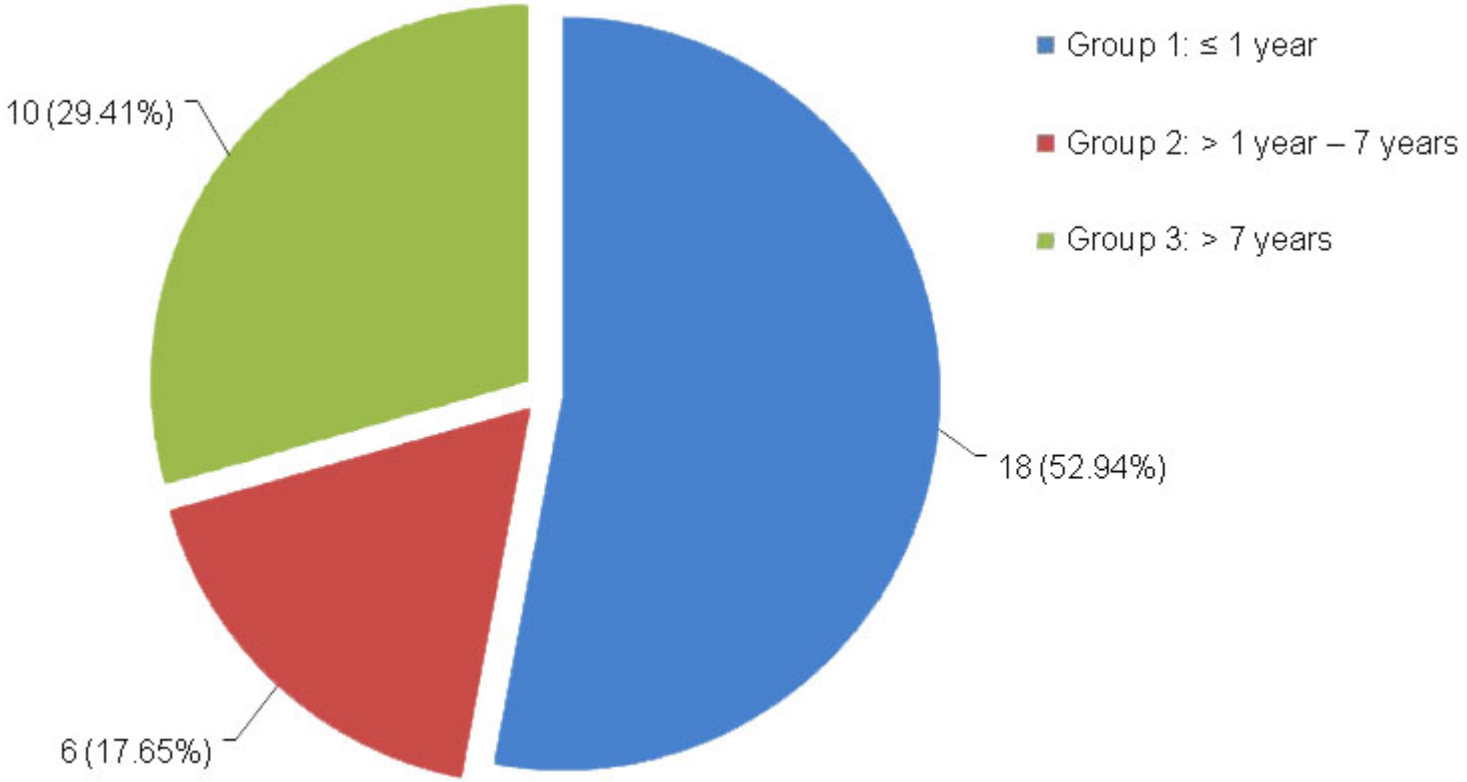

Figure 7: Age distribution of cats which shed *T. gondii* oocysts between June 2007 and December 2008.

Distribution is shown as the proportion relative to the total number (n = 34) of positive cats which shed *T. gondii* in the respective period.

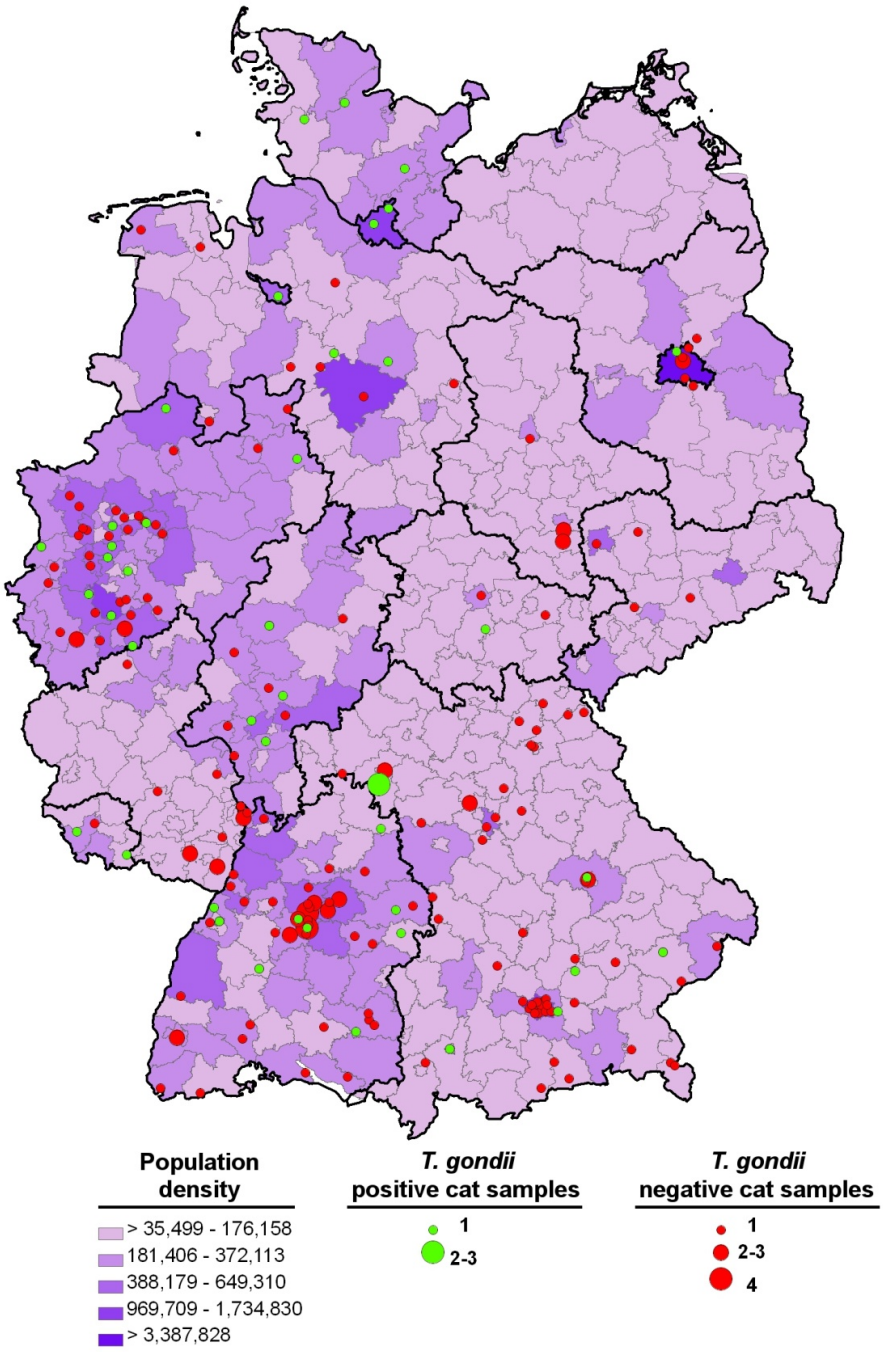

Figure 8: Map of Germany showing where *T. gondii*-positive and -negative samples (June 2007-December 2008) originated from.

Red dots indicate *T. gondii*-negative sample; green dots represent *T. gondii*-positive sample. The size of the dots corresponds to different sample size. Population density (inhabitants/km^2) is shown in purple shading.

3.1.4 Genotyping of *T. gondii* isolated from animal samples

Genotyping was performed with *T. gondii*-DNA isolated from oocysts or tachyzoites (if cultivation in VERO cells after bioassay in mice had been successful) utilising eight unlinked, independent chromosomal marker regions (newSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358 and PK1) and one extra-chromosomal marker region (Apico). An example of a PCR-RFLP analysis using the nine markers is shown in **Figure 9**.

A total of 104 *T. gondii*-positive isolates from Germany were examined. Twenty-two isolates (collected between October 2004 and November 2006) had been only partially characterised in a previous study [Schares *et al.*, 2008b]. All these isolates were now further analysed for the loci c22-8, c29-2, L358, PK1 and Apico [Su *et al.*, 2006]. Oocysts of four isolates which could not be genotyped in the previous study because PCR amplification of the marker regions had failed were now fully typed by PCR-RFLP. The remaining isolates were analysed and genotyped for the first time. The typing results for the feline *T. gondii* isolates at all nine loci are summarised in **Table 17**. The majority (89/104; 85.58%) of these feline *T. gondii* isolates (collected October 2004–November 2006 and June 2007 – December 2010) displayed predominantly type II alleles. Of the 89 samples genotyped as type II at all chromosomal loci, 83 possessed the type I allele (83/104; 79.81%) and six (6/104; 5.77%) displayed the type II allele at the Apico locus (**Figure 10**). One of the 104 isolates, TG-GER13, which could previously not be characterised, was shown to be of type III at all loci (1/104; 0.96%). No clonal type I isolate was detected. Among the 104 isolates, there were four isolates of non-canonical or mixed genotype (4/104; 3.85%). TG-GER02, which had previously been characterised as type II at loci 5' and 3'SAG2, BTUB, SAG3 and GRA6 only, was now shown to contain alleles of types I and II at loci c29-1 and PK1. TG-GER02 was thus identified as a non-canonical, mixed *T. gondii* isolate. TG-GER20, which had not been typed previously, was of type II, except for the

loci Apico and BTUB. This isolate was characterised as type I at the Apico locus, but as types I or II at the BTUB locus. When PCR-RFLP were conducted for BTUB I and BTUB II separately, the isolate revealed the type II allele at the BTUB II locus, but type I and II or III alleles at the locus BTUB I. Isolate TG-GER61 showed a mixed allele pattern only at the Apico locus. Interestingly, isolate TG-GER63 showed a mixture of type II and III alleles at some loci. While only type II patterns could be observed at the loci SAG3 and BTUB, type III alleles were found at GRA6, L358 and Apico loci and a mixture of type II and III alleles at the c22-8, c29-2 and PK1 loci. In addition, a mixture of type I or III and II alleles was found at the newSAG2 locus in the isolate TG-GER63. Ten isolates (10/104; 9.62%) could not be fully genotyped at all nine loci (TG-GER09, 17, 34, 46, 51, 62, 83, 87, 100 and 104). TG-GER09 could only be genotyped at the loci newSAG2, GRA6, c22-8, L358 and Apico. All loci showed alleles of type II. TG-GER17 had alleles of type II at the loci newSAG2, SAG3, BTUB and GRA6, but showed a type I allele at the Apico locus. Isolate TG-GER34 could be genotyped at the loci SAG3, GRA6 and Apico and showed type II alleles at these loci. Isolates TG-GER46 and 52 showed alleles of type II at the loci BTUB, c22-8, L358 and PK1. Additionally, TG-GER51 had a type I allele at the Apico locus. Isolate TG-GER62 shared a type I allele at the Apico locus and a type II alleles at the newSAG2, SAG3, GRA6 and c22-8 loci. In isolate TG-GER83, a type I allele was observed at the Apico locus and a type II allele at the SAG3 locus. The loci c22-8 and c29-2 of TG-GER87 were of type II but the Apico locus showed a type I allele. Alleles at loci newSAG2, BTUB, c22-8, PK1 of isolate TG-GER100 were of type II, but the Apico locus showed a type I allele. No locus could be PCR-amplified from the TG-GER104 isolate. All results are summarised in **Table 17** and **Table 18**.

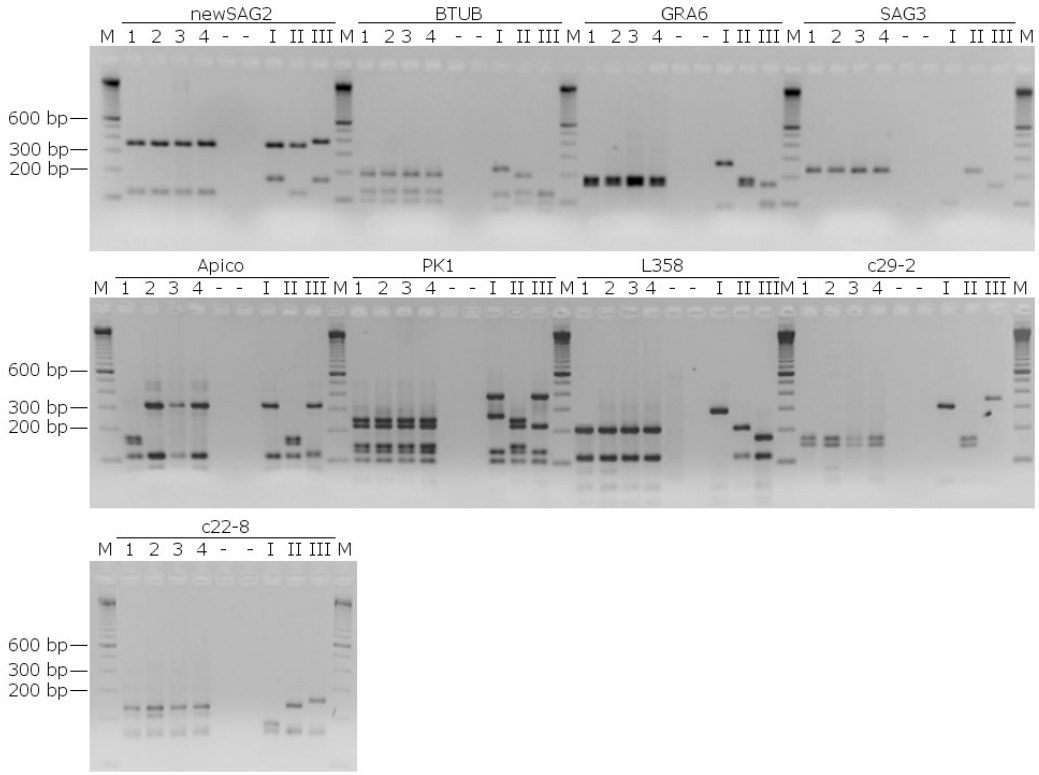

Figure 9: PCR-PFLP polymorphism analyses of the *T. gondii* isolate TG-GER63.

Nine genetic markers (newSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) were utilised. All DNA fragments resulting from specific PCR and DNA endonuclease digestions were resolved in 3% agarose gels. Marker regions: newSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico; M: 100 bp DNA molecular size marker (Invitrogen, Germany); I, III, III: types I (RH), II (ME49) and III (NED); lanes 1–4: *T. gondii* samples.

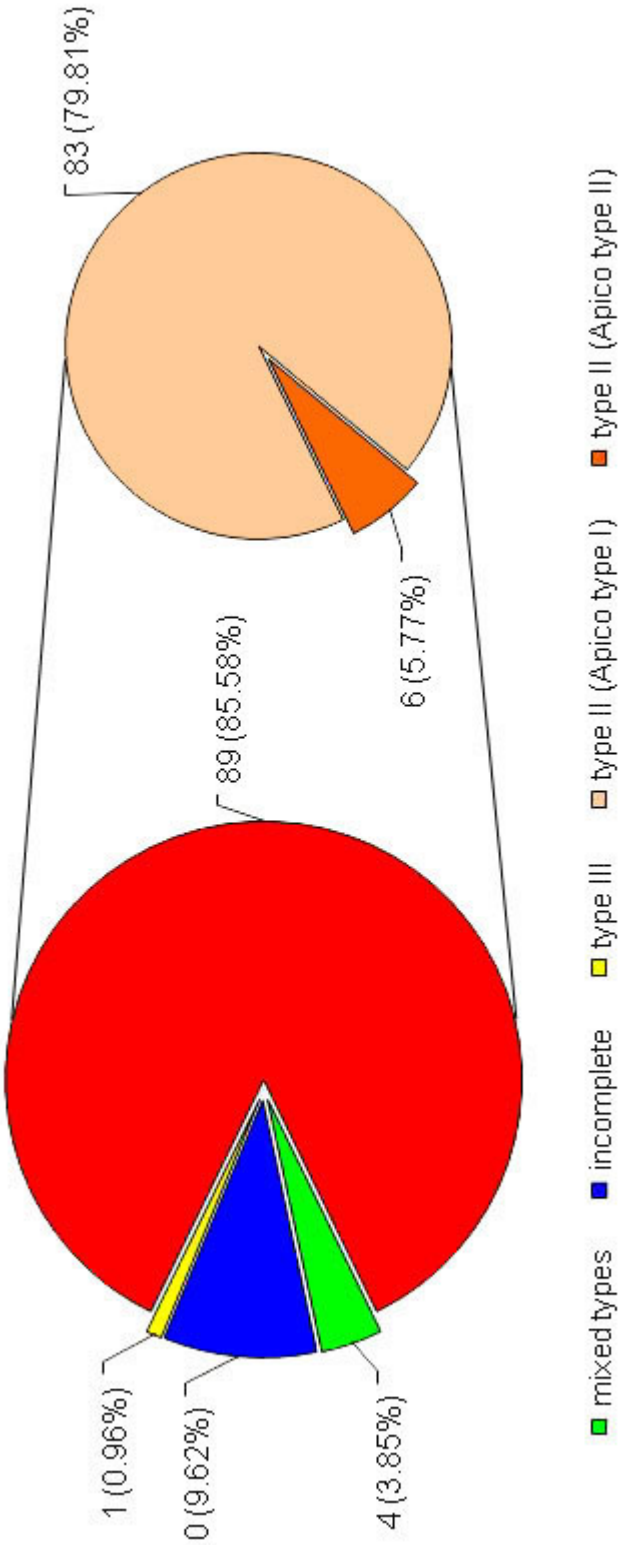

Figure 10: Proportions of different *T. gondii* genotypes observed in oocysts shed by cats from Germany between June 2007 and December 2008.

Table 17: Multilocus genotyping of *T. gondii* isolates from cats from Germany by PCR-RFLP analysis.

nd: no amplification detected; [a]: previously genotyped using markers 3' and 5'SAG2, BTUB, SAG3 and GRA6 only; [*]: DNA from tachyzoites after bioassay in GKO mice

T. gondii isolate	PCR-RFLP genotype (genetic marker)								
	newSAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
RH	I	I	I	I	I	I	I	I	I
ME49	II	II	II	II	II	II	II	II	II
NED	III	III	III	III	III	III	III	III	III
83 (TG-GER03[a], 04[a], 05[a], 06, 08[a], 10[a], 11[a], 12[a], 14[a], 15[a], 16[a], 18[a], 19[a], 21[a], 22[a], 23[a], 24*, 25, 26, 27, 28, 29, 30, 31, 32, 33, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 47, 48, 49*, 52, 53, 54, 55, 56, 57, 58, 59, 60, 64, 65, 66, 67, 69, 71, 72, 73, 74, 75, 77, 79, 80, 81, 82, 84, 86, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 101, 102, 103, 105, 106)	II	II	II	II	II	II	II	II	I
6 (TG-GER07[a], 50, 68, 76, 78, 85)	II	II	II	II	II	II	II	II	II
TG-GER02[a]	II	II	II	II	II	I+II	II	I+II	II
TG-GER09[a]	II	*nd*	*nd*	II	II	*nd*	II	*nd*	II
TG-GER13[a]	III	III	III	III	III	III	III	III	III

Table 17 (continued) *nd*: no amplification detected; [a]: previously genotyped using markers 3' and 5'SAG2, BTUB, SAG3 and GRA6 only; [b]: when analysed separately: BTUB I: type I and II or III allele; BTUB II: type II allele; [*]: DNA from tachyzoites after bioassay in GKO mice

T. gondii isolate	newSAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
TG-GER17[a]	II	II	II	II	*nd*	*nd*	*nd*	*nd*	*nd*
TG-GER20[a]	II	II	I+II [b]	II	II	II	II	II	I
TG-GER34	*nd*	II	*nd*	II	*nd*	*nd*	*nd*	*nd*	*nd*
TG-GER46	*nd*	*nd*	II	*nd*	II	*nd*	II	II	*nd*
TG-GER51	*nd*	*nd*	II	*nd*	*nd*	*nd*	*nd*	*nd*	I
TG-GER61 [*]	*nd*	*nd*	*nd*	*nd*	II	II	*nd*	II	I+II
TG-GER62 [*]	II	II	*nd*	II	II	*nd*	*nd*	*nd*	I
TG-GER63	I or III+II	II	II	III	II+III	II+III	III	II+III	III
TG-GER83	*nd*	II	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	I
TG-GER87	*nd*	*nd*	*nd*	*nd*	II	II	*nd*	*nd*	I
TG-GER100	II	*nd*	II	*nd*	II	*nd*	*nd*	II	I
TG-GER104	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

3.2 Genetically and biological characterisation of isolate TG-GER63

Interestingly, sample TG-GER63 showed more than one allele at several loci (**Table 17** and **Figure 11**) and also contained sporulated oocysts. Such unusual allele patterns could only appear if at least two genetically different *T. gondii* oocysts existed in the sample. Since TG-GER63 had been isolated from a single feline faecal sample, it seemed most likely that all oocysts contained in the sample originated from a single cat. However, this also meant that the cat must have harboured at least two genetically different *T. gondii*.

It followed that sexual recombination of *T. gondii* may have taken place in the cat which might have generated not only the parental *T. gondii* genotypes by selfing but also many genetically recombinant or re-assorted *T. gondii*. Even more remarkable is the presence of type III and type II alleles in the oocysts sample, since type III was shown to be rare in cats in Germany. Two different scenarios may explain such an allele pattern in TG-GER63: (i) there were only type II and type III *T. gondii* in this single faecal sample, but differential PCR amplification of different loci revealed the mixed allele patterns found at some but not all loci (ii) all observations of a single type-specific allele observed at a single locus (e.g. at loci SAG3, BTUB, GRA6, L358 and Apico) were not due to differential PCR amplification, but the real genetic situation was reflected in the PCR-RFLP. Therefore, all observations of more than one type-specific allele observed at a single locus (i.e. at the loci newSAG2, c22-8, c29-2 and PK1) were not the result of differential PCR amplification, but the real genetic situation reflecting a mixture of genetically different *T. gondii* oocysts in the sample. This would lead to the conclusion that the sample had consisted of many genetically different non-canonical types of *T. gondii*. To test these two hypotheses, the single oocyst sample had to be separated into the components it comprised of.

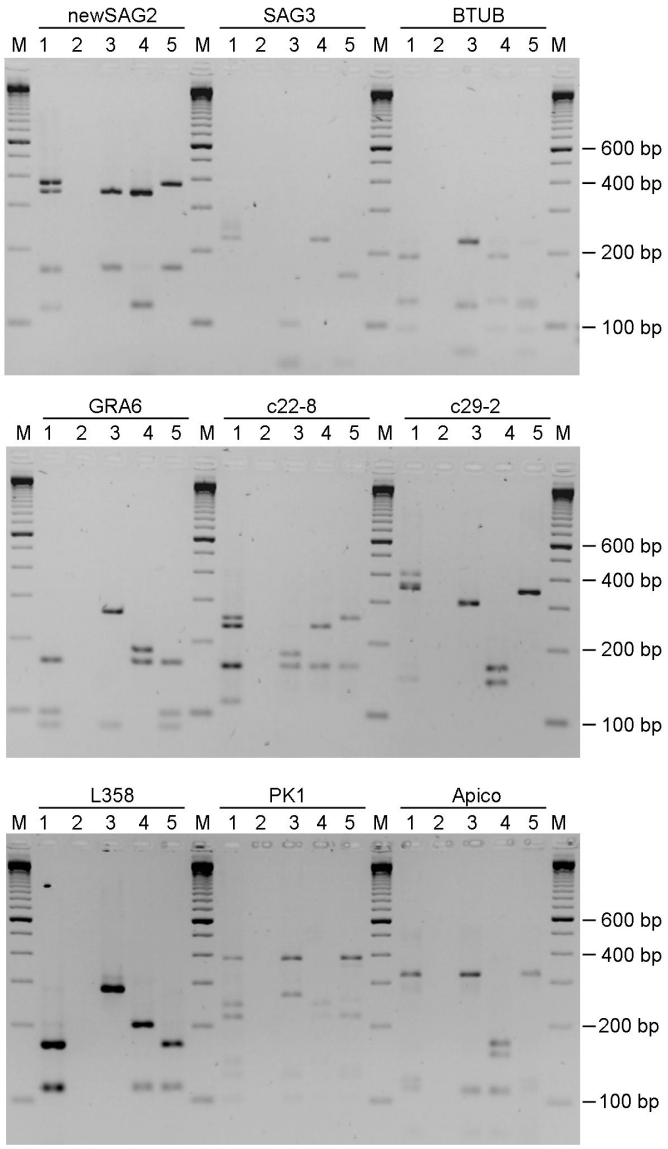

Figure 11: PCR-RFLP polymorphism analyses of the *T. gondii* isolate TG-GER63.

Nine genetic markers (newSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) were utilised. DNA fragments were resolved in 3% agarose gels. M: 100 bp DNA molecular size marker (Invitrogen, Germany); 1: Isolate TG-GER63; 2: negative control (water); 3, 4 and 5: reference strains type I (RH), type II (ME49) and type III (NED), respectively.

3.2.1 Bioassay of TG-GER63 oocysts and mouse infections

To identify different individual genotypes in the oocyst field sample TG-GER63, two GKO-mice were orally infected with 10^4 sporulated oocysts from TG-GER63. Both mice showed first clinical signs at eight d.p.i. and were sacrificed accordingly. *T. gondii* tachyzoites were isolated from those mice and cultured on VERO cells (K119/1 and K119/2). Genotyping by PCR-RFLP of the two isolates revealed a mixture of type II and III alleles at several individual loci (**Table 18**). The pattern was similar but not identical with the one observed in the original oocyst isolate TG-GER63 (**Table 17**). Tachyzoites harvested from the K119/1 cell culture were used to infect five BALB/c mice (B136/1, B136/2, B136/3, B136/4 and B136/5) which died 20, 22, 23, 21 or 21 d.p.i., respectively. *T. gondii*-infected VERO cell cultures, one from each mouse, were established using peritoneal washings. All *T. gondii* tachyzoites isolated from infected GKO and BALB/c mice showed a mixture of type II and type III alleles at different loci (**Table 18**).

Table 18: Multilocus genotyping by PCR-RFLP of *T. gondii* clones.

K: *T. gondii* obtained from infected GKO-mouse; B: *T. gondii* obtained from infected BALB/c-mouse

T. gondii isolate	Inoculum	PCR-RFLP genotype (genetic marker)								
		newSAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
RH		I	I	I	I	I	I	I	I	I
ME49		II	II	II	II	II	II	II	II	II
NED		III	III	III	III	III	III	III	III	III
TG-GER63		I or III+II	II	II	III	II+III	II+III	III	II+III	III
K119/1	Oocysts (TG-GER63)	III	II	III	II+III	II+III	II+III	II+III	II	III
K119/2	Oocysts (TG-GER63)	III	II	III	III	II+III	III	III	III	III
B136/1	Tachyzoites (K119/1)	II+III	III	III	II+III	II+III	III	II+III	II+III	III
B136/2	Tachyzoites (K119/1)	II+III	III	III	II+III	III	III	II+III	II+III	III
B136/3	Tachyzoites (K119/1)	II+III	III	III	II+III	III	III	II+III	II+III	III
B136/4	Tachyzoites (K119/1)	II+III	III	II+III	II+III	III	III	II+III	II+III	III
B136/5	Tachyzoites (K119/1)	II+III	III	III	II+III	II+III	III	II+III	II+III	III

3.2.2 Cloning of *T. gondii* isolates

In order to separate the components of the mixed *T. gondii* isolate from each VERO cell culture, limiting dilutions were carried out. After each round, the generated isolates were genotyped. If the isolate still resembled a mixed *T. gondii* culture, subcloning by limiting dilution was carried out. Thus, several rounds of limiting dilution resulted in the isolation of 45 distinct *T. gondii* clones from mixed *T. gondii* VERO cell cultures. A flow diagram illustrating this process containing all clones generated by this method is shown in **Figure 12**. Most clones displayed a combination of type II and III alleles at several different loci. Clones displaying an identical allele combinations over all examined loci were thus grouped accordingly (**Table 19**). In total, five genotypically different *T. gondii* clone groups were identified, based on their PCR-RFLP allele pattern. *T. gondii* clones in four groups showed a non-canonical allele pattern that have never been described in any intermediate or definitive host species before (groups 2–5). Group 1 contained sixteen clones displaying type III alleles at all loci. All twenty-two clones of group 2 showed alleles of type III at all loci except for a type II allele at PK1 (Chr. VI). Clones within group 3 displayed type II alleles at the BTUB (Chr. IX) and c29-2 (Chr. III) loci with all remaining loci shared type III alleles. All clones in group 4 displayed type III alleles except for the type II allele at newSAG2 (Chr. VIII), c22-8 (Chr. Ib), L358 (Chr. V) and PK1. Group 5 contained one clone which was almost identical with members of group 4 except for a type I allele at the Apico locus.

While type II alleles were initially observed at the SAG3 locus (Chr. XII) in cell cultures infected with *T. gondii* of K119/1 and K119/2, none of the subsequently isolated clones displayed a type II allele at that locus. Mixed cell cultures isolated from K119/1 always showed a mixture of type II and III alleles at the GRA6 locus (Chr. X), but no type II allele was observed at this particular locus in any resulting clone.

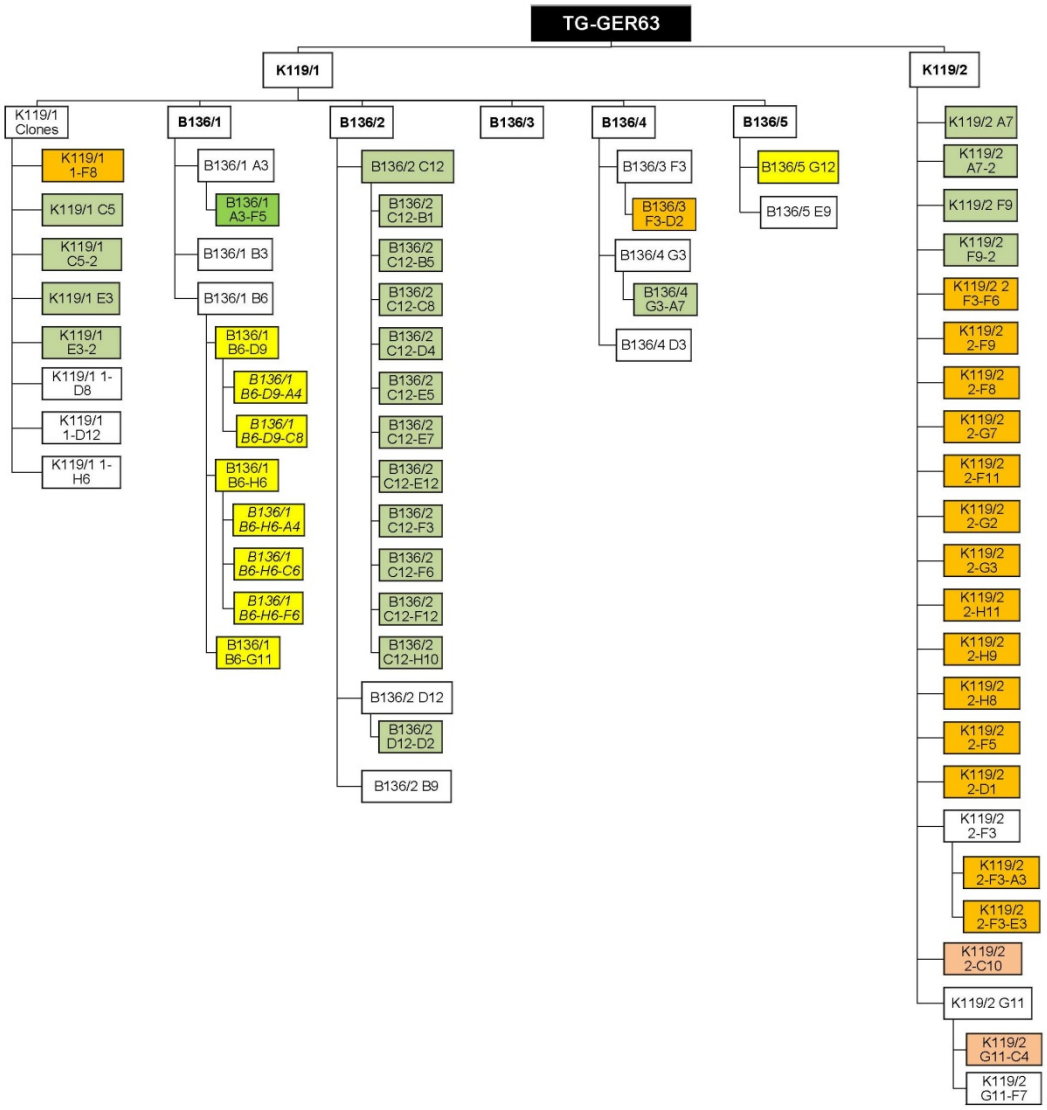

Figure 12: Flow diagram of *T. gondii* clones generated from oocyst isolate TG-GER63 by limited dilution.

Bold letters indicate bioassay (B: BALB/c mouse; K: GKO mouse). White shading indicates isolates with a mixed allele pattern at at least one locus. Coloured shadings indicate clones with a single allele at a given locus. *T. gondii* clones shaded with the same colour had the same PCR-RFLP allele pattern at all nine loci.

Table 19: Multilocus genotyping of individual *T. gondii* clones after limiting dilution revealed five groups of *T. gondii*.

Genotyping of *T. gondii* clones isolated from *in-vitro* cell cultures of *T. gondii* by limiting dilution. Typing was performed by PCR-RFLP analysis. *T. gondii* clones were grouped according to their allele pattern. K: *T. gondii* cell culture clone obtained from infected GKO-mouse; B: *T. gondii* cell culture clone obtained from infected BALB/c-mouse

Designation	Origin (number of clones)	PCR-RFLP genotype (genetic marker)								
		newSAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
Group 1 (16)	K119/2 (10), B136/4 (4), K119/1 (1), B136/5 (1)	III	III	III	III	III	III	III	III	III
Group 2 (22)	B136/2 (13), K119/1 (4), K119/2 (4), B136/4 (1)	III	III	III	III	III	III	III	II	III
Group 3 (2)	K119/2 (2)	III	III	II	III	III	II	III	III	III
Group 4 (4)	B136/1 (3), B136/5 (1)	II	III	III	III	II	III	II	II	III
Group 5 (1)	B136/4 (1)	II	III	III	III	II	III	II	II	I

3.2.3 Virulence of isolated *T. gondii* clones

To determine the virulence of the clones isolated from TG-GER63, up to two clones from each clone group were randomly chosen to determine their *in-vivo* virulence in BALB/c mice using different tachyzoite doses of each clone as indicated in **Table 20**. All mice used in these experiments were tested for seroconversion by immunoblot analysis upon death or at the end of the experiment to confirm infection (30 d.p.i.). All mice inoculated with *T. gondii* seroconverted except for mice that died before 10 d.p.i. and three mice inoculated with only 10 parasites.

Representatives of clone groups 1 and 3 were of low virulence in mice. Infecting mice with 10^6 tachyzoites i.p. did not result in death in many mice. Representatives of clone group 2 showed an intermediate ($LD_{50} > 10^2$–10^4 tachyzoites) or high ($LD_{50} > 10$–100 tachyzoites) virulence phenotype in mice. All representatives of clone groups 4 and 5 were highly virulent ($LD_{50} < 100$ tachyzoites) in mice. Survival data for all clones at each dose are shown in **Figure 13** and **Figure 14**. Mice infected with 10^6 *T. gondii* from groups 4 and 5 died within 14 days of infection. In contrast, animals infected with low-virulent clones survived the challenge with 10^6 *T. gondii* parasites. Interestingly, challenge with *T. gondii* containing a mixed population of *T. gondii* clones (K119/1 and K119/2) killed mice at 27 and 30 d.p.i. (**Figure 13A**). Survival data of infection with 10^4 *T. gondii* varied greatly between clones. The time of death did not seem to be correlated with the genotype of the parasite. Although the *T. gondii* clone with the highest overall virulence (B136/1 B6-H6) killed mice earlier at this dose, *T. gondii* clones of group 2 (intermediate and highly virulent *T. gondii* clones K119/ 2 A7 and B136/2 C12) showed higher virulence than the highly virulent clones of B136/5 G12 and B136/1 A3-F5 (**Figure 13B**). The results of the high parasite number challenge were confirmed when only 100 parasites were used. Again, *T. gondii* clones B136/5 G12 and B136/1 A3-F5 showed the lowest virulence considering time of death and mortality (60%) of the parasite challenge. In contrast, *T. gondii* clones

B136/2 C12 and B136/1 B6-H6 were the most virulent clones at this dose. Interestingly, both highly virulent *T. gondii* clones had different allele patterns (**Figure 14A**). At the low-dose challenge, *T. gondii* clone B136/1 B6-H6 was the most virulent clone followed by *T. gondii* clone B136/1 A3-F5. Both clones had a very similar allele pattern with only the Apico locus being different. Again, *T. gondii* clones B136/5 G12 and B136/2 C12 were the least virulent ones among the high-virulent clones (**Figure 14B**).

Table 20: Variation of *in-vivo* virulence of *T. gondii* clones in BALB/c mice between and within clone groups.

Five BALB/c mice were each infected with 10^6, 10^4, 10^2, 10 and 0 tachyzoites i.p. per clone. *nd*: not done

Designation	Clone group	% Mortality in mice (No. dead/No. infected)					LD_{50}	Virulence
		10^6	10^4	10^2	10	0		
K119/2 2-H8	1	20 (1/5)	*nd*	*nd*	*nd*	0 (0/5)	$>10^6$	low
K119/2 2 F3-A3	1	20 (1/5)	*nd*	*nd*	*nd*	0 (0/5)	$>10^6$	low
K119/2 A7	2	100 (5/5)	100 (5/5)	0 (0/5)	*nd*	0 (0/5)	$>10^2$–10^4	intermediate
B 136/2 C12	2	100 (5/5)	100 (5/5)	100 (5/5)	20 (1/5)	0 (0/5)	>10–100	high
K119/2 2-C10	3	0 (0/5)	*nd*	*nd*	*nd*	0 (0/5)	$>10^6$	low
K119/2 G11-C4	3	0 (0/5)	*nd*	*nd*	*nd*	0 (0/5)	$>10^6$	low
B136/5 G12	4	100 (5/5)	100 (5/5)	60 (3/5)	20 (1/5)	0 (0/5)	>10–100	high
B136/1 B6-H6	4	100 (5/5)	100 (5/5)	100 (5/5)	100 (2/2)	0 (0/5)	<10	high
B136/1 A3-F5	5	*nd*	100 (5/5)	60 (3/5)	50 (1/2)	0 (0/5)	10	high

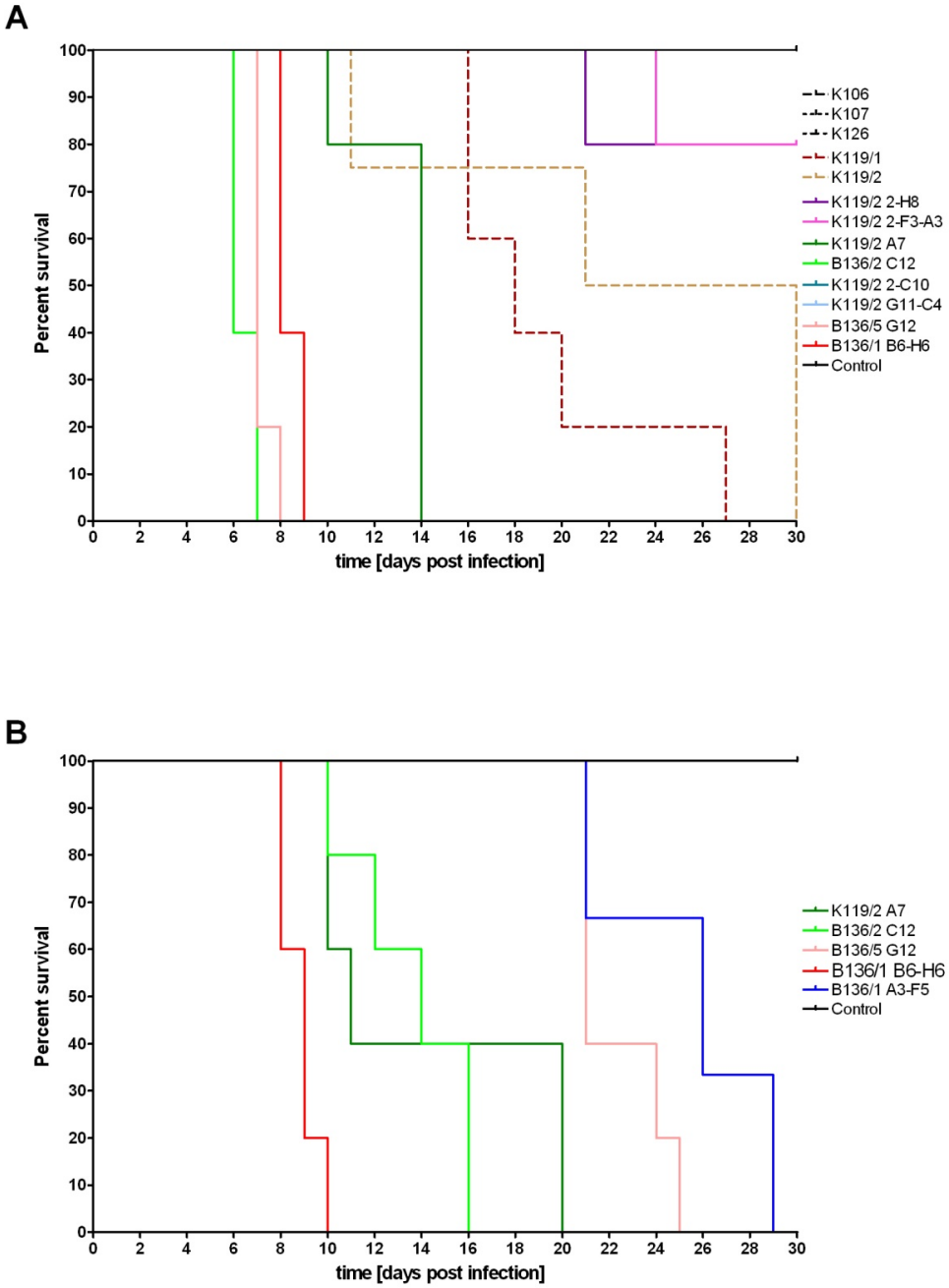

Figure 13: Survival of BALB/c mice infected with 10^6 and 10^4 genetically different *T. gondii* clones.

Five BALB/c mice per *T. gondii* clone were each infected with (A) 10^6 tachyzoites i.p. or (B) 10^4 tachyzoites i.p. of different *T. gondii* clones. *T. gondii* clones are indicated each by a different coloured line. *T. gondii* clones that did not show any virulence after infection with the higher dose were not used in subsequent experimental infections using lower doses.

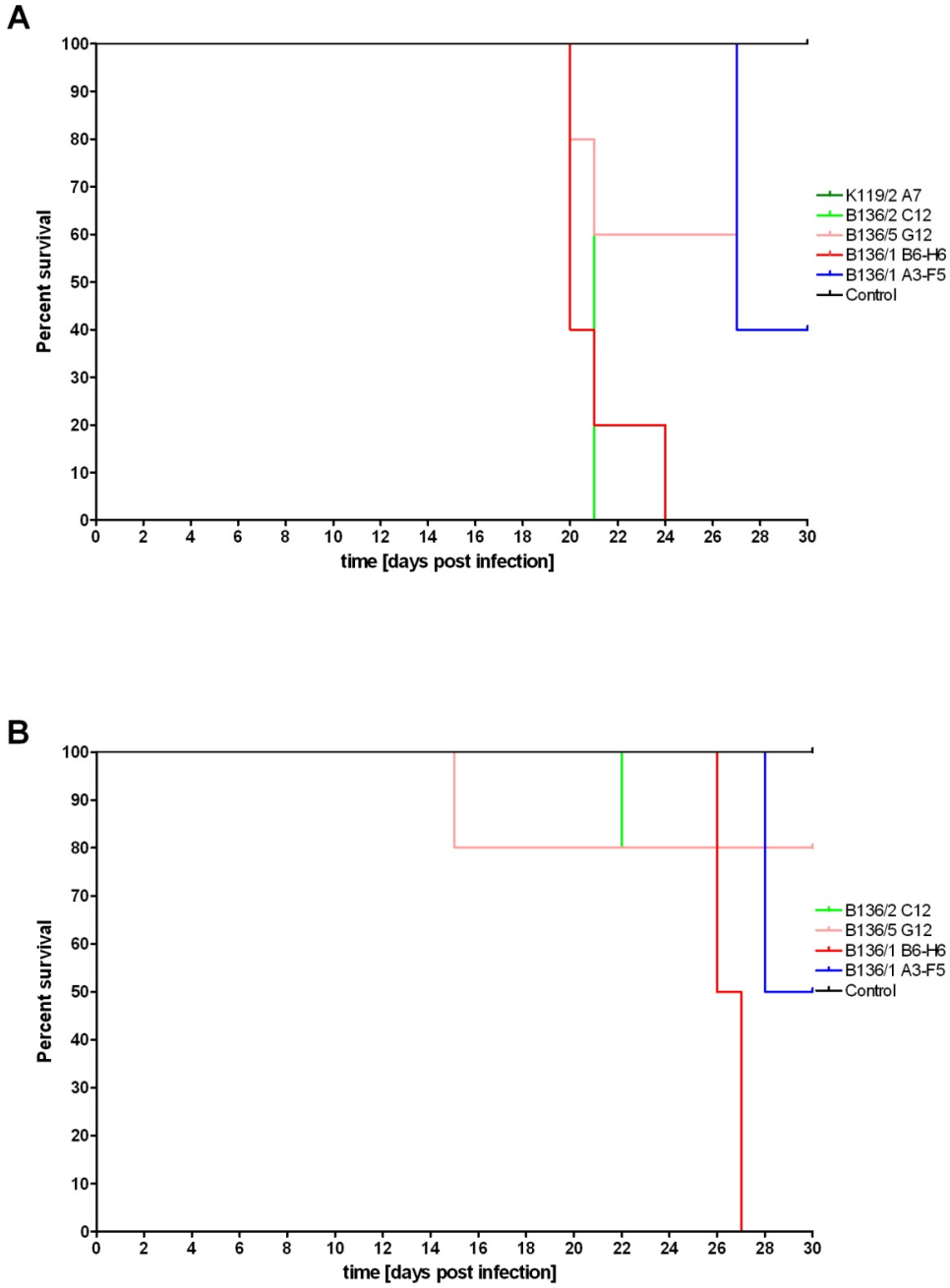

Figure 14: Survival of BALB/c mice infected with 100 and 10 genetically different *T. gondii* clones.

Five BALB/c mice per *T. gondii* clone were each infected with (A) 100 tachyzoites i.p. or (B) 10 tachyzoites i.p. of different *T. gondii* clones. *T. gondii* clones are indicated each by a different coloured line. *T. gondii* clones that did not show any virulence after infection with the higher dose were not used in subsequent experimental infections using lower doses.

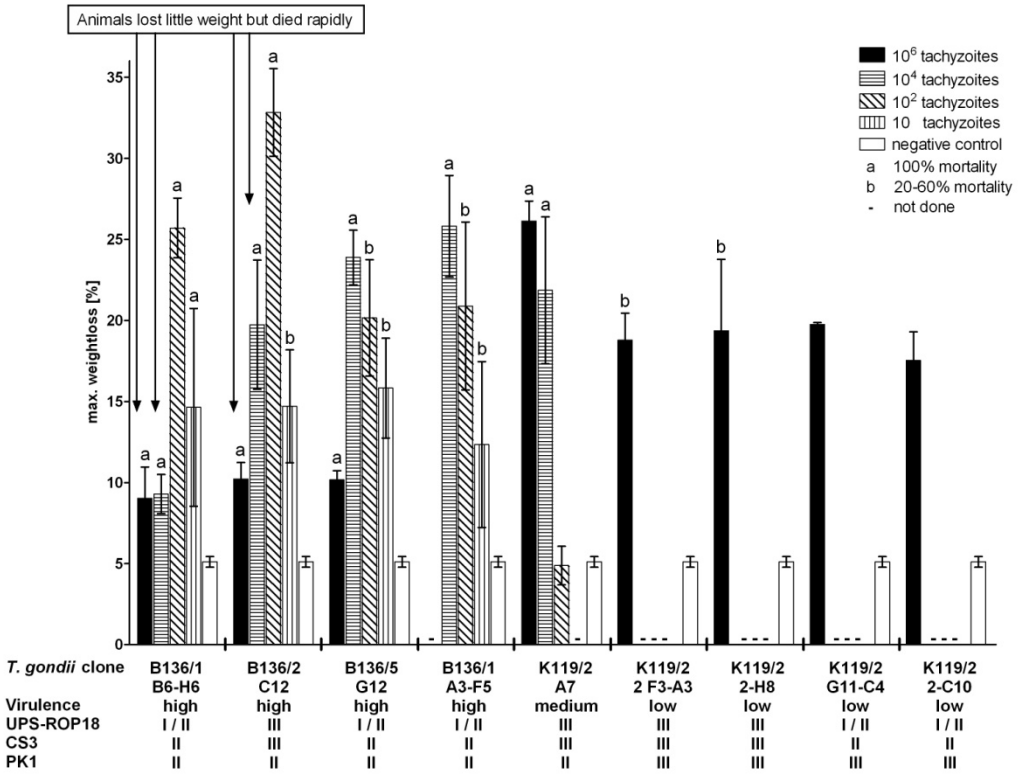

Figure 15: Maximum weight loss of BALB/c mice upon experimental infection with genetically different *T. gondii* clones.

Five animals per group were infected with 10^6, 10^4, 10^2 or 10 *T. gondii* tachyzoites i.p. each. Data shown are maximum weight loss (in per cent), virulence markers: UPS-ROP18 [Khan et al., 2009] and CS3 [Pena et al., 2008] and genetic marker PK1 [Su et al., 2006].

All mice lost weight after infection with *T. gondii*, irrespective of the genotype or the infection dose. Weight loss and dose of infection were positively correlated, except for high-dose challenges with virulent clones which killed the mice rapidly. In contrast, mice infected with low-virulent *T. gondii* clones and negative control animals lost only up to 5.00% of weight. Infection with high doses (10^6 and 10^4 parasites) of high-virulent clones (groups 2 and 4) led to weight losses of only up to 10.00%, but the animals died within 2–3 days after the onset of disease. However, mice infected with lower doses of high-virulent clones had lost more than 15.00% of their weight when they died, in some cases even more than

30%. Some animals showed a rapid decrease from less than 20.00% to 30.00% weight loss within one day (**Figure 15**).

Detailed analysis of maximal weight losses between the clones showed that infection with *T. gondii* was almost always statistically significantly correlated with weight loss as compared to control groups. However, this phenomenon could only be associated with the non-canonical genotypes. Type II wild-type isolates K106, K107 and K126 did not show a significant difference in terms of weight loss as compared to the control group (**Figure 16A**). Despite a statistically significant maximum weight loss compared with the control animals, infection with *T. gondii* clones K119/2 2-H8 did not result in any death of the infected mice and the weight of the infected animals increased at the end of the experiment. Statistical differences in terms of maximum weight loss in the high virulence groups are also misleading since these animals died rapidly within a few days. The weight loss was therefore not as pronounced as in challenge infections with 10^4 tachyzoites (**Figure 16B**). Challenge with 10^4 virulent *T. gondii* clones resulted in statistically significant weight loss in all infected mouse groups as compared to control animals (**Figure 16B**). Mice infected with 10^2 *T. gondii* tachyzoites of clone K119/2 A7 showed hardly any weight loss. Surprisingly, the virulent *T. gondii* clone B136/1 A3-F5 showed not statistically significant differences in terms of weight loss at this dose. However, mice in that group all died late but lost a considerable amount of weight within only two days after the onset of symptoms. All other mice infected with virulent *T. gondii* clones showed significant weight loss as compared to the control animals (**Figure 17A**). Upon challenge with 10 tachyzoites, the extremely highly virulent *T. gondii* clone B136/1 B6-H6 caused statistically significant weight losses in infected mice. Interestingly, animals infected with B136/5 G12 also showed higher weight loss than observed in control animals (**Figure 17B**).

When analysing weights of organs from infected and control animals, only spleens showed statistically significant differences to control mice. The weights of

lungs, brains or kidneys of infected animals were not significantly different compared to those of control mice (data not shown). Almost all mice infected with 10^6 *T. gondii* clones suffered from splenomegaly as a result of acute infection with T. *gondii* (**Figure 18A**). Spleens of all infected mice were considerably enlarged. Interestingly, mice infected with the high-virulent *T. gondii* clone B136/5 G12 did not show statistically significant enlarged spleens. However, mice in that particular group died extremely fast shortly after infection. In mice infected with 10^4 tachyzoites, spleens were enlarged in almost all mouse groups regardless of the *T. gondii* genotype they were infected with (**Figure 18B**). All mice infected with 100 tachyzoites of high-virulent *T. gondii* showed statistically significant enlarged spleens (**Figure 19A**). At the lowest dose of infection (10 *T. gondii* tachyzoites), only animal groups with the highest number of survivors (*T. gondii* clones B136/2 C12 and B136/5 G12) showed significant differences in their spleen weight as compared to control animals. Mice infected with 10 tachyzoites of high-virulent *T. gondii* clones (B136/1 B6-H6 and B136/1 A3-F5) showed no differences compared with the control animals (**Figure 19B**).

When the route of *in-vivo* isolation was analysed, it became apparent that all highly mouse-virulent clones had been passaged through BALB/c mice prior to *in-vitro* isolation, while all clones isolated after a GKO-mouse passage were of low or intermediate mouse virulence.

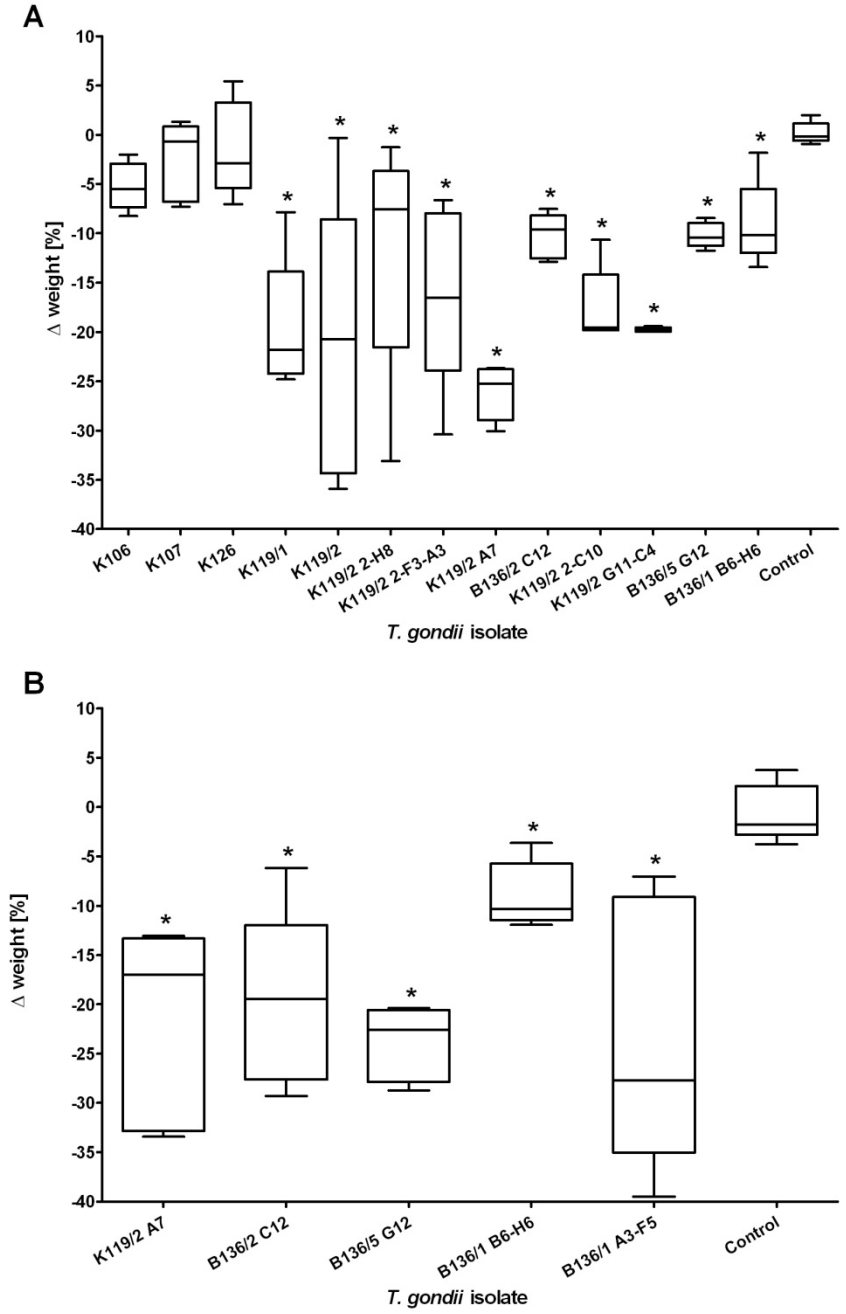

Figure 16: Maximum changes in weight of BALB/c mice infected with (A) 10^6 or (B) 10^4 *T. gondii* tachyzoites i.p.

Five BALB/c mice per *T. gondii* clone were each infected with (A) 10^6 tachyzoites i.p. or (B) 10^4 tachyzoites i.p. Whiskers: 95% CI; *: statistically significant differences ($P < 0.05$, Mann-Whitney U test) to control mice.

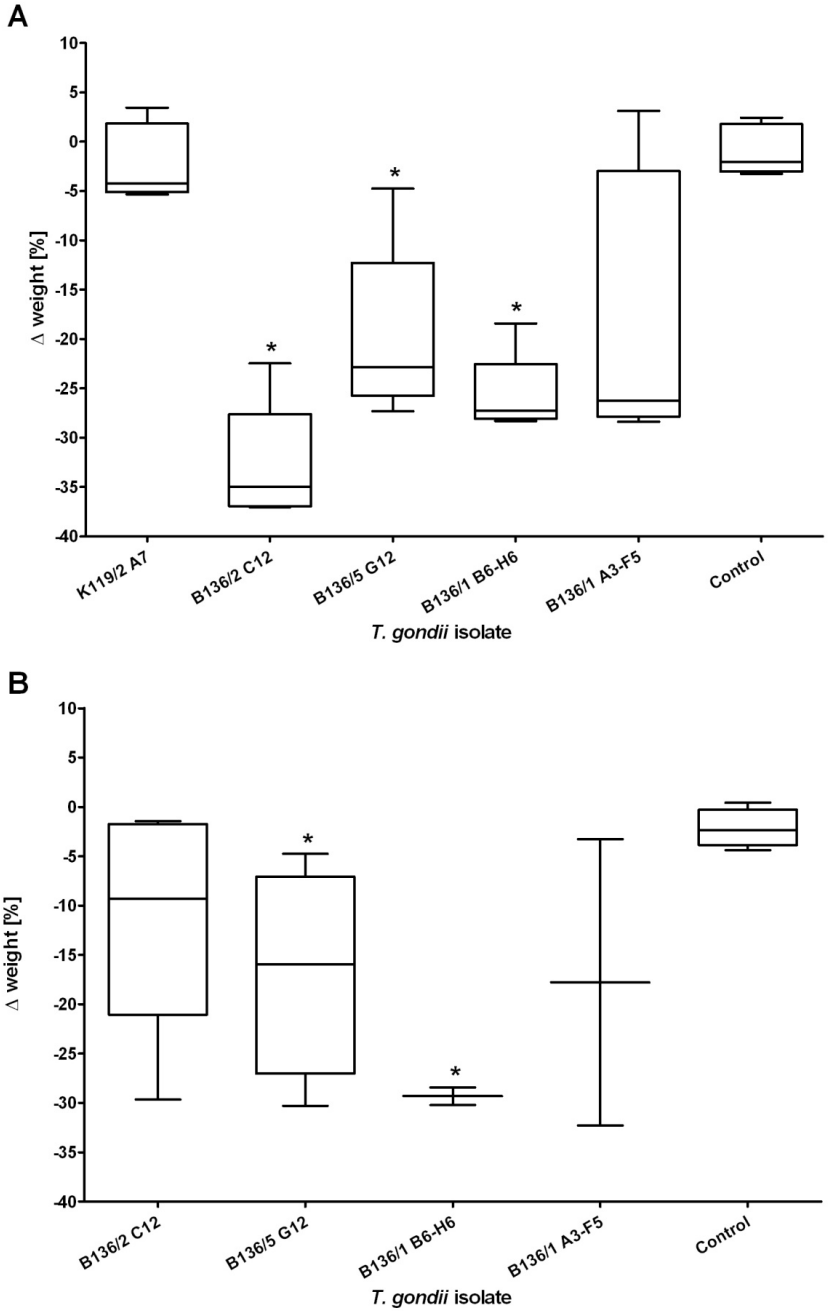

Figure 17: Maximum changes in weight of BALB/c mice infected with (A) 100 or (B) 10 *T. gondii* tachyzoites i.p.

Five BALB/c mice per *T. gondii* clone were each infected with (A) 100 tachyzoites i.p. or (B) 10 tachyzoites i.p. Whiskers: 95% CI; *: statistically significant differences ($P < 0.05$, Mann-Whitney U test) to control mice.

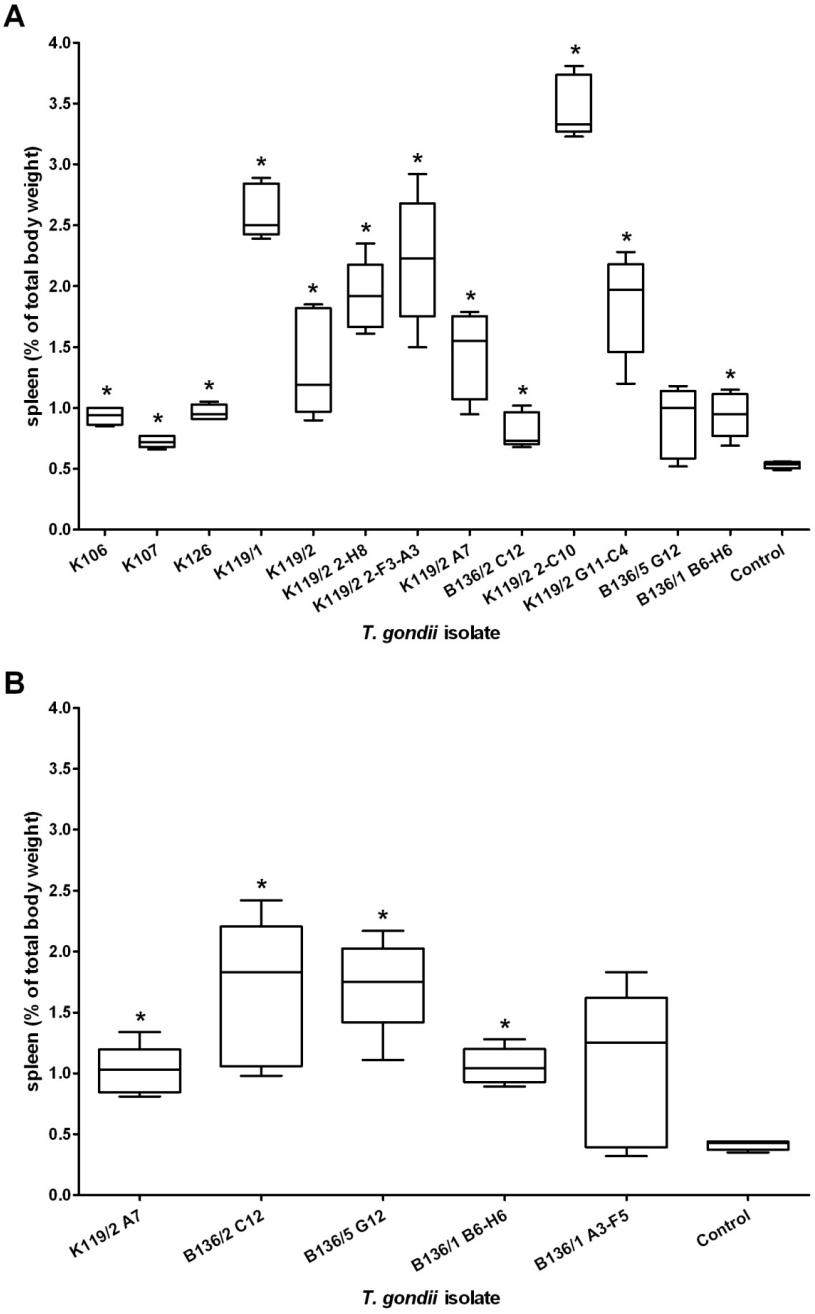

Figure 18: Maximum changes in spleen weight of BALB/c mice infected with (A) 10^6 or (B) 10^4 *T. gondii* tachyzoites i.p.

Five BALB/c mice per *T. gondii* clone were each infected with (A) 10^6 tachyzoites i.p. or (B) 10^4 tachyzoites i.p. Whiskers: 95% CI; *: statistically significant differences ($P < 0.05$, Mann-Whitney U test) to control mice.

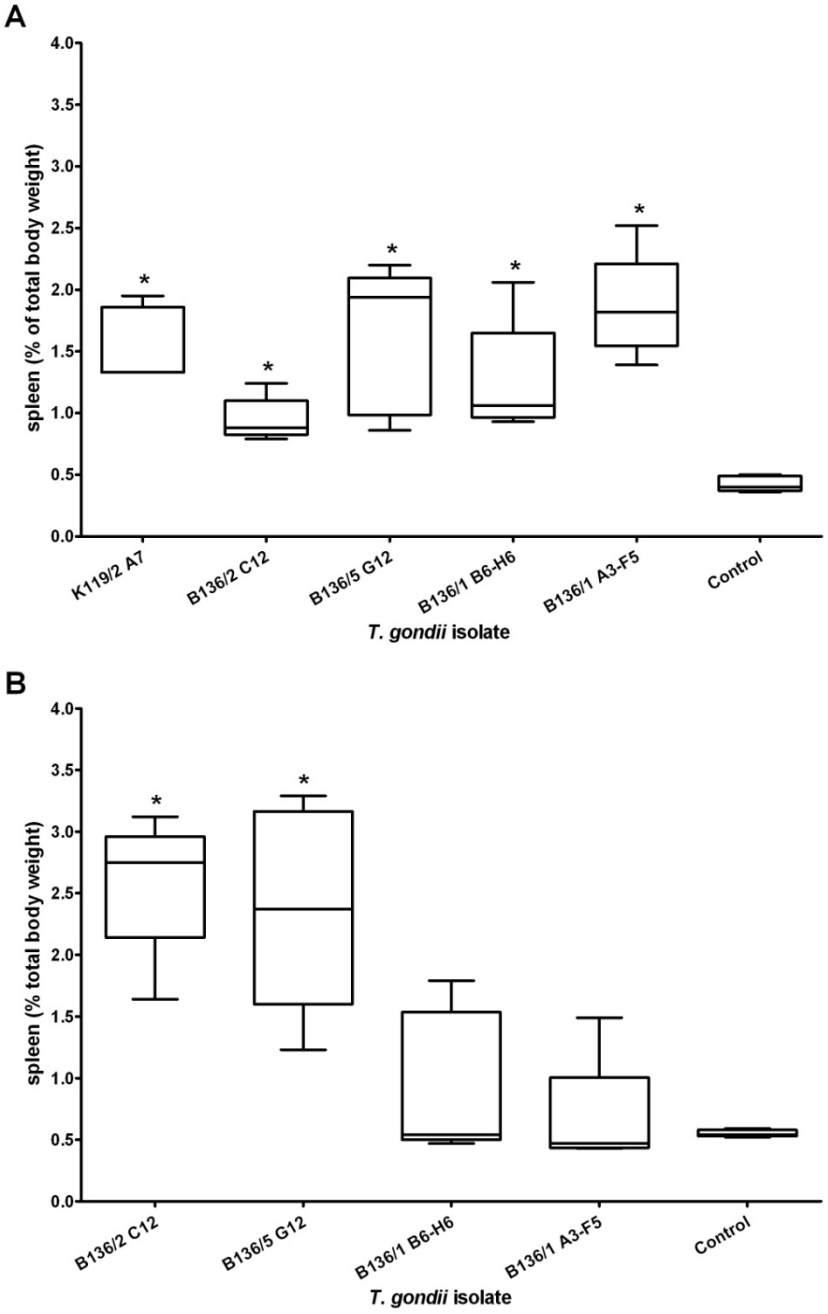

Figure 19: Maximum changes in spleen weight of BALB/c mice infected with (A) 100 or (B) 10 *T. gondii* tachyzoites i.p.

Five BALB/c mice per *T. gondii* clone were each infected with (A) 100 tachyzoites i.p. or (B) 10 tachyzoites i.p. Whiskers: 95% CI; *: statistically significant differences ($P < 0.05$, Mann-Whitney U test) to control mice.

3.3 Virulence markers

The typing results for putative mouse virulence markers were compared and tested for agreement with the actual *in-vivo* virulences obtained from experimental infections. Predictions using the virulence marker CS3 corresponded perfectly with the predictions of UPS-ROP18 (**Table 21**). UPS-ROP18 was absent (type I/II) in all representatives of clone groups 4 and 5 ($LD_{50} < 100$ tachyzoites) which correctly predicted their high virulence for mice. UPS-ROP18 was present (UPS-ROP18III*) in representatives of clone group 1 ($LD_{50} > 10^6$ tachyzoites) which correctly predicted their low virulence for mice. However, UPS-ROP18 was absent in representatives of clone group 3 ($LD_{50} > 10^6$ tachyzoites), thus predicting high virulence for mice, which is in contrast to their low virulence for BALB/c mice. Representatives of clone group 2 showed intermediate ($LD_{50} > 10^2$ tachyzoites) and high ($LD_{50} > 10$ tachyzoites) virulence for BALB/c mice despite the presence of UPS-ROP18 and CS3 type III, which have been proposed as predictors of low mouse virulence [Khan *et al.*, 2009].

The clones described here showed a positive correlation between virulence and the PK1 allele as determined by PCR-RFLP. All mouse-virulent clones had a type II allele at the PK1 locus, whereas non-virulent clones had a type III allele present at this locus.

DNA sequencing of the UPS-ROP18 was carried out to identify possible sequence differences between virulent and non-virulent UPS-ROP18III* and to determine the UPS-ROP18 type in *T. gondii* clones groups 3–5 identified as UPS-ROP18I* or ROP18II*. All *T. gondii* clones that had the UPS-ROP18 absent were of UPS-ROP18II* as indicated by an additional 44 bp repeat which is absent in UPS-ROP18I*. However, no difference were detected between the UPS-ROP18III* sequences of *T. gondii* clones with high and low virulences for BALB/c-mice **(**details described in the **Appendix)**.

Table 21: Mouse virulence of different *T. gondii* clones is not associated with the presence or absence of the UPS-ROP18 per se.

T. gondii clones were analysed for virulence using published virulence markers UPS-ROP18 [Khan et al, 2009] and CS3 [Pena et al., 2008].

Designation	Clone group	PK1	Virulence marker		Predicted virulence	LD_{50}	Observed virulence
			CS3	UPS-ROP18			
K119/2 2-H8	1	III	III	III	low	$>10^6$	low
K119/2 2 F3-A3	1	III	III	III	low	$>10^6$	low
K119/2 A7	2	II	III	III	low	$>10^2$–10^4	intermediate
B 136/2 C12[f]	2	II	III	III	low	>10-–100	high
K119/2 2-C10	3	III	I / II	II	high	$>10^6$	low
K119/2 G11-C4	3	III	I / II	II	high	$>10^6$	low
B136/5 G12	4	II	I / II	II	high	>10–100	high
B136/1 B6-H6	4	II	I / II	II	high	<10	high
B136/1 A3-F5	5	II	I / II	II	high	10	high

3.4 Analysis of additional chromosomal markers

To investigate whether *T. gondii* clones of different allelic patterns were the result of a recombination event or of a re-assortment of chromosomes in the definitive host, additional chromosomal markers were studied in one or two clones from each clone group (**Table 22**). No allelic type differences were observed between the genetic markers used for genotyping and the additional chromosomal markers in *T. gondii* clones of clone groups 1 (K119/2 2-H8, K119/2 2 F3-A3) and 2 (K119/2 A7, B136/2 C12). Interestingly, *T. gondii* clones of clone group 3 (K119/2 2-C10, K119/2 G11-C4) showed alleles of different types on Chr.V, while type III alleles were observed on this chromosome using the genetic marker L358 and type II allele was detected using the genetic marker AK22. Clones in clone group 4 (B136/5 G12, B136/1 B6-H6) and 5 (B136/1 A3-F5) had a type III allele at locus c29-2, but a type II allele at locus AK97, both of which are located on Chr.III. Furthermore, a type II allele was observed at the nSAG2 locus, but a type III allele at the L375 locus, both of which are located on Chr.VIII.

Table 22: Multilocus genotyping of *T. gondii* clones used in virulence study by applying additional chromosomal markers.

Typing was performed by PCR-RFLP analysis. *T. gondii* clones were grouped according to their allele pattern. a: [Su *et al.*, 2006]; b: [TGMD]; §: evidence for sexual recombination

Chr.	Marker	*T. gondii* clones								
		Group 1		Group 2		Group 3§		Group 4§		Group 5§
		K119/2 2-H8	K119/2 2-F3-A3	K119/2 A7	B136/2 C12	K119/2 2-C10	K119/2 G11-C4	B136/5 G12	B136/1 B6-H6	B136/1 A3-F5
Ib	c22-8[a]	III		III		III		II		II
	AK16[b]	III		III		III		II		II
III§	c29-2[a]	III		III		II		**III**		**III**
	AK97[b]	III		III		II		**II**		**II**
V§	L358[a]	III		III		**III**		II		II
	AK22[b]	III		III		**II**		II		II
VI	PK1[a]	III		II		III		II		II
	L53[b]	III		II		III		II		II
VIII§	newSAG2[a]	III		III		III		**II**		**II**
	AK53[b]	III		III		III		**III**		**III**
IX	BTUB[a]	III		III		II		III		III
	L375[b]	III		III		II		III		III
X	GRA6[a]	III		III		III		III		III
	AK69[b]	III		III		III		III		III
XII	SAG3[a]	III		III		III		III		III
plastid	Apico[a]	III		III		III		III		I

3.5 Analysis of human samples

A total of nine DNA samples from patients with ocular toxoplasmosis (TG-hssGER01–09) and two DNA samples from patients with congenital toxoplasmosis (TG-hssGER10–11) were available for analysis.

Of the nine samples from patients with ocular toxoplasmosis only five (TG-hssGER01, 03, 04, 06 and 08) were weakly positive for *T. gondii*-DNA using conventional PCR detecting the 529 bp repeat region of *T. gondii* (TOX5/Tox-8 primer). Therefore, the *T. gondii*-DNA content in the samples was deemed to be very low. Because of the small amount of DNA in the samples a multiplex-PCR was used to amplify the nine genetic marker regions for genotyping. As expected, only partial genotyping was possible for samples that were positive in the conventional *T. gondii*-PCR. In the sample TG-hssGER01, only three *T. gondii* type II alleles were detected at loci c22-8, L358 and Apico. Interestingly, in sample TG-hssGER03, alleles of either *T. gondii* type I or III were detected at loci GRA6 and PK1. In samples TG-hssGER04 and 07, the highest number of loci could be PCR-amplified. The yield was low, however, with only 3/9 loci. In both cases, only alleles of *T. gondii* clonal type II were observed. While sample TG-hssGER04 was successfully genotyped at the loci newSAG2, SAG3 and PK1, in TG-hssGER07 loci BTUB, GRA6 and L358 could be PCR-amplified. All results are summarised in **Table 23**.

All *T. gondii*-DNA samples from patients with congenital toxoplasmosis could be completely typed for all selected markers. Both samples showed alleles of type II except for the Apico locus where a type I allele was observed as summarised in **Table 24**.

Another sample containing vitreous liquid of a patient with ocular toxoplasmosis was supplied by Prof. Pleyer (Charite, Berlin). The sample was centrifuged at 1,100 x g; 7 min without brake. The supernatant was removed and the pellet resuspended with 100 µl DMEM. Half of the resulting solution was used to isolate parasite DNA using the Nucleospin Tissue kit (Macherey-Nagel). The remaining 50 µl were filled up to 500 µl with DMEM medium and used to inoculate a GKO-mouse i.p.. At three months p.i. no symptoms were observed in the inoculated mouse. Serum collected at 90 d.p.i. was seronegative for *T. gondii*. Furthermore, DNA isolated from the sample was PCR-negative for *T. gondii*.

Table 23: Multilocus genotyping of *T. gondii*-DNA isolated from patient with ocular toxoplasmosis.

Genotyping was performed by PCR-RFLP analysis. *nd*: not detected

T. gondii isolate	Clinical status	PCR-RFLP genotype (genetic marker)								
		newSAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
RH		I	I	I	I	I	I	I	I	I
ME49		II	II	II	II	II	II	II	II	II
NED		III	III	III	III	III	III	III	III	III
TG-hssGER01	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	II	*nd*	II	*nd*	II
TG-hssGER02	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
TG-hssGER03	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
TG-hssGER04	ocular toxoplasmosis	II	II	*nd*	*nd*	*nd*	*nd*	*nd*	II	*nd*
TG-hssGER05	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
TG-hssGER06	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
TG-hssGER07	ocular toxoplasmosis	*nd*	*nd*	II	II	*nd*	*nd*	II	*nd*	*nd*
TG-hssGER08	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
TG-hssGER09	ocular toxoplasmosis	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Table 24: Multilocus genotyping of *T. gondii*-DNA isolated from patients with congenital toxoplasmosis.

Genotyping was performed by PCR-RFLP analysis. *nd*: not detected

T. gondii isolate	Clinical status	PCR-RFLP genotype (genetic marker)								
		newSAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
RH		I	I	I	I	I	I	I	I	I
ME49		II	II	II	II	II	II	II	II	II
NED		III	III	III	III	III	III	III	III	III
TG-hssGER10	congenital toxoplasmosis	II	II	II	II	II	II	II	II	I
TG-hssGER11	congenital toxoplasmosis	II	II	II	II	II	II	II	II	I

4 Discussion

4.1 *T. gondii* genotypes in cats in Germany

Most studies on *T. gondii* in Germany have concentrated on serological investigations in livestock animals so far. Together with results on oocyst shedding and genotyping of *T. gondii* in cats the work presented here tries to give a more comprehensive overview of *T. gondii* genotypes in cats in Germany.

The aim of this study was to characterise the genotypes of *T. gondii* oocysts obtained from feline faecal samples. To achieve this, 82 new *T. gondii* oocyst isolates from feline faecal samples were collected and analysed in addition to 22 isolates which were already available from a previous study [Schares *et al.*, 2008b]. Among the resulting total of 104 isolates there were four isolates with non-canonical or mixed *T. gondii* genotypes. To the best of our knowledge, *T. gondii* isolates of such non-canonical and mixed infections have never been detected in Europe before and have never been observed in the faeces of naturally infected cats worldwide. For the first time, genetically different non-canonical *T. gondii* of high and low mouse virulence excreted by a single naturally infected feline host were found and characterised. However, there was no evidence of atypical *T. gondii* or isolates carrying atypical alleles shed by cats from Germany.

Although 18,259 feline faecal samples were examined and statistically analysed, this study cannot be regarded as population-wide or representative in terms of its spatial distribution (e.g. urban or rural), cat ownership (e.g. private or farm) or cat feeding (e.g. commercial feed, mice, birds) because cat owners and veterinarians submitted these samples to diagnose diarrhoea (in about 60.00% of the cases) or for routine testing. However, no temporal constraints were placed on this study; therefore it is representative of the temporal distribution of cats shedding *T. gondii* oocysts.

The results show that for the period June 2007–December 2008 only 0.25% of the feline faecal samples were *T. gondii*-positive. Interestingly, the proportion of *T. gondii*-positive samples collected between January and June (0.09%) was significantly lower than between July and December (0.31%). This may indicate that the risk for intermediate hosts (including humans) to acquire a *T. gondii*-infection via oocysts could vary over the year, being highest in the second half of the year. Such seasonality may at least in part be due to the availability of infected prey (e.g. rodents, birds) to cats. If the risk of infection for prey, e.g. via oocyst contamination in the environment, and an increased number of prey early in the second half of the year is assumed, the chance of a cat to eat infected prey and to shed oocysts will also increase in this part of the year. However, this seasonality only applies to cats shedding *T. gondii* oocysts.

Due to data protection we cannot draw any conclusion on whether the studied cats were fed raw or not appropriately treated meat products (in contrast to tinned food) or whether they were allowed to roam outside. This is important to take into account when analysing seasonality.

Cats that roam outside may be exposed to additional risks of acquiring *T. gondii* by hunting and eating infected birds or small mammals (such as rodents). However, the seroprevalence of *T. gondii* in mice from the Federal State of Brandenburg was shown to be very low (0.00%) [Herrmann *et al.*, 2012]. Pigeons from Portugal had a 62.50% *T. gondii* prevalence [Waap *et al.*, 2008]. More importantly, the seroprevalences reported in rodents vary greatly. The *T. gondii*-DNA prevalence in Austria was shown to be only 0.70% in *Microtus arvalis* [Fuehrer *et al.*, 2010]. In Switzerland, the *T. gondii* seroprevalence was also found to be low, with 3.00% *Microtus arvalis* and 5.00% of *Arvicola terrestris* being seropositive for this parasite [Reperant *et al.*, 2009]. In contrast, shrews (*Sorex spp.*; 60.00%), voles (*A. terrestris*; 22.00%) and moles (*Talpa europaea*; 39.00%) from France showed high seroprevalences [Afonso *et al.*, 2007]. Interestingly, a high *T. gondii* prevalence in rodents was also found by PCR in *Mus musculus* in the United

Kingdom that were captured as part of a pest control programme in domestic dwellings in Manchester [Hughes *et al.*, 2006].

Since there was no information on the behaviour of cats analysed in this study, it is difficult to identify the sources of infection for cats in Germany. Studies on cats in urban areas also suggest that there were sites where cats preferred to defecate [Afonso *et al.*, 2008]. Therefore, hot spots in terms of sites contaminated with *T. gondii* oocysts may exist. Other sources of *T. gondii* infections for cats could therefore also include oral uptake of oocyst-contaminated soil, food sources or water. Another possible but unlikely source of infection may be earthworms which have been described as parantenic hosts of *T. gondii* [Afonso *et al.*, 2007; Ruiz and Frenkel, 1980].

A recent study on *T. gondii* in foxes from Germany showed that the seroprevalence was up to 85.00% [Herrmann *et al.*, 2012]. This indicates that *T. gondii* must be present in German wild life and that the sylvatic life cycle of *T. gondii* is quite complex, possibly involving many different mammals and birds capable of transmitting the parasite. Oocysts deposited on soil also do not stay but may be washed off. Recent studies using *T. gondii* oocyst surrogates showed that landscape changes caused by man and/or nature facilitate run-off and thus increase the transport of oocysts. This effect was shown to be intensified by extensive dry periods and may increase with increasing temperatures [Shapiro *et al.*, 2010a; Shapiro *et al.*, 2010b].

A large proportion (47.06%) of *T. gondii* shedding cats was over 12 months old. Moreover, a number of cats older than seven years (10/34; 29.42%) were found to shed *T. gondii* oocysts. This finding confirms an earlier report indicating that older cats shed *T. gondii* oocysts more often than previously thought [Schares *et al.*, 2008b]. Lack of data regarding the habits of the older cats and the conditions the cats were kept in made it impossible to draw any conclusions on the reason why older cats shed oocysts. Reports show that *T. gondii* positive cats can

shed oocysts again even years after their first shedding event. Superinfection could be another possibility why older cats were found to shed *T. gondii* oocysts. [Dubey, 1995; Frenkel and Smith, 1982; Lappin *et al.*, 1996]. Of course, it is also possible that the old oocyst-shedding cats observed were infected for the first time in their life.

Most *T. gondii* samples originated from areas with a high population density. However, this may only reflect areas where most samples came from. This was shown by the fact that the random sample of cats negative for oocyst shedding that was investigated in this study had also been submitted from these areas. As far as we know, all faecal samples were submitted to VetMed Labor GmbH either by the owner or the veterinarian. Therefore we can assume that the cats sampled were most likely domestic cats. However, this does not rule out any contact of the cats with the sylvatic cycle of *T. gondii* in Germany.

In this study, previous work [Schares *et al.*, 2008b] on *T. gondii* oocysts by faecal samples over a period of over three years was extended. *T. gondii* isolates were all characterised using nine molecular markers (newSAG2, BTUB, GRA6, SAG3, c22-8, c29-2, L358, PK1 and Apico). When comparing the results of the previous study with the work presented here, it is evident that the use of a single locus or even four loci does not give sufficient information to determine the *T. gondii* genotype unambiguously. Isolates previously described as type II were found to contain non-type II alleles at additional loci using another five genetic markers for PCR-RFLP. The correctly designated non-canonical isolates provide important information regarding the genetic diversity of *T. gondii* in Germany.

The majority of *T. gondii* oocysts shed by cats had type II-specific allele patterns. This confirms previous findings indicating that human congenital toxoplasmosis in Europe is mainly caused by type II *T. gondii* [Ajzenberg *et al.*, 2002a; Aspinall *et al.*, 2003; Fuentes *et al.*, 2001; Honore *et al.*, 2000; Howe *et al.*, 1997; Nowakowska *et al.*, 2006]. However, most of our type II-like isolates were of type

I at the Apico locus with only a few having a type II pattern at this locus. When the Apico marker was first described, the type II isolate DEG which had been isolated from a French patient with latent congenital toxoplasmosis [Darde *et al.*, 1992], was also characterised as type I at the Apico marker region [Su *et al.*, 2006]. *T. gondii* with type II alleles at all chromosomal loci but with type I at the Apico locus have also been observed by others [Berger-Schoch *et al.*, 2011a; Dubey and Jones, 2008; Prestrud *et al.*, 2008]. The apicoplast is a secondary endosymbiont and inherited via the maternal line [Ferguson *et al.*, 2005]. Therefore, our observation would only fit to the model that clonal type I has arisen from just a few crosses between ancestral type II parents and other ancestral strains (paternal) [Saeij *et al.*, 2006] if two lineages of type II strains exist (one with a type I and the other with a type II apicoplast) and only the one with a type I apicoplast contributed to the rise of type I. However, to confirm the validity of the Apico PCR-RFLP marker used in the present study and to determine the maternal inheritance of the many isolates, more sequence data on the apicoplast genomes of clonal types I and II strains are needed.

Only one *T. gondii* isolate from feline faecal samples was of type III and none of type I. Considering that 38,576 cat samples were examined in total (June 2007–December 2008), the confidence intervals indicate that the true prevalence of *T. gondii* type I in our target cat population is lower than 0.008%. Therefore, it may be concluded that type I and type III are relatively rare in cats from Germany or even absent (type I).

Other European countries, such as Spain, Portugal or Poland, reported *T. gondii* clonal types genetically different from type II in humans [Ajzenberg *et al.*, 2005; Grigg *et al.*, 2001b] and animals such as chickens [Dubey *et al.*, 2008b], pigs [de Sousa *et al.*, 2006] and pigeons [Waap *et al.*, 2008]. This situation may be explained by an uneven spatial distribution of *T. gondii* types I and III within Europe. Geographical boundaries may also influence this uneven distribution. For

example, high mountain ranges separate the Iberian Peninsula from the rest of Europe.

The first early maritime trading activities took place between South America and the Iberian Peninsula, where non-type II *T. gondii* are observed. However, some of the largest modern-day ports are situated in the Netherlands and Germany and increased international and European trade importing meat and pets should have established non-type II in Germany. Surprisingly, non-canonical types of *T. gondii* were identified in chickens in the neighbouring country Poland [Dubey *et al.*, 2008b]. Another study suggests that non-canonical *T. gondii*-DNA was found in cattle from Switzerland [Berger-Schoch *et al.*, 2011b]. This may indicate that non-type II *T. gondii* could circulate in some intermediate host species (domestic or wild animals). However, *T. gondii* was not isolated in these studies but only DNA samples from tissue analysed. Without typing a *T. gondii* isolate, one cannot be sure that the tissue did not harbour different types of *T. gondii* while only one of the alleles was PCR-amplified from the sample. Another study on *T. gondii* in foxes from Germany found predominately type II but there was evidence of mixed *T. gondii*-type infection and non-canonical types circulating in wild animals in Germany [Herrmann *et al.*, 2012]. Moreover, the *T. gondii* genotype patterns observed in oocyst-shedding cats probably reflect only a fraction of the whole spectrum of genotypes present in Germany. In this country, genotyping of *T. gondii* was carried out for isolates and *T. gondii*-DNA of tissues from cats, foxes and humans so far. To get a clearer picture of the population structure of *T. gondii*, more intermediate host species need to be investigated. Of particular interest could be infected migrating birds that may introduce new genotypes from countries which have a more genetically diverse *T. gondii* population structure, such as Brazil, into Germany.

Among all *T. gondii* isolates, we found only four with non-canonical and mixed genotypes, if the results for the Apico marker are not taken into consideration. The occurrence of mixed genotypes, i.e. the presence of more than one geno-

type at a single locus, may indicate the presence of a mixed population in the respective oocyst sample. The presence of a mixed population can be explained by sexual recombination or re-assortment of chromosomes during a sexual cross after simultaneous infection of a cat with *T. gondii* isolates of different genotypes. Successive experimental infection with two strains of different genotypes was shown to be possible in mice [Araujo *et al.*, 1997; Dao *et al.*, 2001]. This suggests that recombination between different *T. gondii* types in a cat after ingesting only one intermediate host harbouring two different *T. gondii* genotypes would be possible.

The sample TG-GER63 showed a pattern consisting of a mixture of type II- and III-specific alleles scattered over several loci and chromosomes which would be consistent with this interpretation. However, sexual recombination is believed to occur very seldom in nature while self-mating of *T. gondii* seems to be one of the predominant routes of reproduction. This is regarded as one of the reasons for the clonal population structure of *T. gondii* observed in North America and Europe [Grigg and Sundar, 2009]. There is increasing evidence that sexual recombination may play an important role in the evolution and diversification of *T. gondii* strains in nature [Ajzenberg *et al.*, 2004; Dubey, 2008; Dubey and Jones, 2008; Dubey *et al.*, 2008c; Grigg and Suzuki, 2003], especially in South America [Pena *et al.*, 2008], Africa [Lindström Bontell *et al.*, 2009; Lindström *et al.*, 2008; Mercier *et al.*, 2010; Velmurugan *et al.*, 2008] and Asia [Dubey *et al.*, 2007h; Dubey *et al.*, 2008b; Zhou *et al.*, 2009; Zhou *et al.*, 2010]. Mixed genotypes can be induced experimentally [Saeij *et al.*, 2006; Taylor *et al.*, 2006] by crossing different *T. gondii* types *in-vitro* and *in-vivo*. In countries where domestic felids are absent, wild felids, such as Jaguars in French Guiana, may serve as definitive hosts [Demar *et al.*, 2008]. This indicates that there might be both, a domestic and a wild (sylvatic) cycle. Observations of mixed genotypes in indigenous prey, such as free-ranging chickens in South America [Dubey *et al.*, 2005b; Dubey *et al.*, 2005c; Dubey *et al.*, 2006a; Dubey *et al.*, 2006b; Dubey *et al.*, 2006c; Dubey *et al.*, 2006d; Dubey *et al.*, 2007a; Dubey *et al.*, 2007f; Dubey *et al.*, 2008e; Pena *et*

al., 2008], deer in the USA [Dubey and Jones, 2008; Dubey *et al.*, 2008f], cougars, raccoons and skunks in the Boreal forests of Canada [Dubey *et al.*, 2007g; Dubey *et al.*, 2008c] indicate the importance of the sylvatic cycle as a driving force for genetic diversity in *T. gondii*.

4.2 Non-canonical *T. gondii* have high virulences in BALB/c mice

In this study, a number of genetically different, non-canonical *T. gondii* clones were isolated from a single faecal sample of a naturally infected cat. To our knowledge, this is the first report showing that sexual recombination in a natural infected cat may have led to the generation of genetically different *T. gondii*. More importantly, these non-canonical *T. gondii* types show differences in virulence for BALB/c mice. We identified high-virulent as well as low-virulent *T. gondii*. Virulence for BALB/c mice differed not only between genotype groups (nine PCR-RFLP marker regions) but interestingly also between *T. gondii* clones within the same genotype group.

The only previous report with indications for a natural sexual recombination or re-assortment of chromosomes in *T. gondii* came from Uganda [Lindström *et al.*, 2008]. Sequencing the reported natural type II/III *T. gondii* (TgCkUg2) revealed that this isolate has arisen via chromosome sorting and not via interchromosomal recombination, possibly through a single sexual cross of type II and type III *T. gondii*. Interestingly, this isolate caused a significantly higher parasite density in mice as compared to a related type II isolates but did not reach the comparatively high parasite density of a type III isolate. It was concluded that this virulence potentiation was achieved by a sexual cross, and that the increased virulence was due to the identified virulence loci [Lindström Bontell *et al.*, 2009]. Examination of another *T. gondii* isolates from chickens and cats in a waterborne toxoplasmosis outbreak in Brazil found several *T. gondii* genotypes which may have been the result of a sexual recombination event. However, this study was based on RFLP and MS genotyping only [Wendte *et al.*, 2010].

Furthermore, *T. gondii* possesses virulence genes that show an altered biological potential in recombinant strains [Sibley and Ajioka, 2008]. Certain alleles of the genes ROP18 (located on Chr. VIIa) and ROP16 (located on Chr. VIIb) have virulence-potentiating properties [Saeij *et al.*, 2006; Taylor *et al.*, 2006]. Analysis of *T. gondii* strains from different geographical regions of the world discovered that only three major alleles of this gene (ROP18I*, ROP18II* and ROP18III*) were evident world-wide, corresponding to types I, II and III, respectively [Khan *et al.*, 2009]. More importantly, the majority of South American isolates share alleles of the ROP18I* gene which is associated with high virulence in mice. Isolates of the ROP18III* type had an upstream region (UPS-ROP18) which was absent in isolates of the ROP18II* and ROP18I* type. Virulence data of the isolates showed that almost all isolates of the ROP18III* type were avirulent in mice whereas absence of UPS-ROP18 (ROP18I* and ROP18II*) was associated with higher virulence in mice. However, there were notable exceptions to this rule. Although UPS-ROP18 was present, and thus the isolates should have been of low virulence, the *T. gondii* isolates P89 and CAST were in fact shown to be highly virulent for mice [Khan *et al.*, 2009]. In the current study, representatives of *T. gondii* clone group 2 (B136/2 C12 and K119/2 A7) seem to confirm this. Although these isolates were of the ROP18III* ("avirulent") type, they caused high mortality in BALB/c mice. To confirm that the UPS-ROP18 region was really present, the UPS-ROP18 region was sequenced in B136/2 C12 and K119/2 A7. Both virulent *T. gondii* clones were confirmed to be of the UPS-ROP18III* type and the sequence was shown to be identical to the "avirulent" *T. gondii* type III (VEG) reference strain. According to the virulence markers, *T. gondii* of the ROP18I* or the ROP18II* type should be mouse-virulent [Khan *et al.*, 2009]. However, the present study does not fully agree with this hypothesis since *T. gondii* isolates with a low virulence for BALB/c mice (clone group 3; K119/2 2-C10 and K119/2 G11-C4) were all of the ROP18II* type as confirmed by DNA sequencing. It was therefore concluded that the published virulence markers [Khan *et al.*, 2009] can only give limited information regarding the mouse-virulence of different *T. gondii*

clones. The presence or absence of UPS-ROP18 in different *T. gondii* isolates does not seem to be associated with mouse virulence. Other factors must therefore confer virulence in type I *T. gondii* parasites. It was shown that there is variation in the expression of ROP18 between the different *T. gondii* clonal types. While expression of ROP18 is increased in *T. gondii* type I and II, it was shown to be lower in *T. gondii* type III [Saeij *et al.*, 2006].

Recent investigations into the action of ROP18 have further elucidated its function in mouse virulence. ROP18 is a member of the ROP2 gene family expressed during invasion of *T. gondii*. It is one of the contributors to resistance of type I strains to the IRG system in mice. When "avirulent" *T. gondii* strains actively invade cells they form a PVM and within minutes IFN-γ-stimulated cells express IRG proteins (Irgb6 and Irga6) that accumulate at the PVM. As a result, the PVM starts to disrupt and to disintegrate [Pawlowski *et al.*, 2011; Virreira *et al.*, 2011]. However, only type I strains were shown to inhibit this process. The ROP18 protein of type I was able to directly phosphorylate mouse IRG proteins at two conserved threonine residues in an important nucleotide binding domain. As a result, the GTPase is catalytically inactivated and thus unable to locate and to bind to the PVM. The authors concluded that only *T. gondii* expressing the active form of ROP18, like type I strain do, will evade IRG-killing. It was also found that type II ROP18 only differs from type I in 22 amino acids, whereas type III differs from type I in 78 amino acids. However, since the expression level of ROP18 in type III *T. gondii* is greatly reduced, this might be another reason for the greatly reduced virulence of type III in mice [Steinfeldt *et al.*, 2010]. Forward genetics has confirmed the mode of action of ROP18. *T. gondii* type III expressing active type I ROP18 were shown to be mouse virulent in contrast to the wild-type type III. The level of secreted ROP18 on the PVM was shown to be inversely correlated to Irgb6 staining at the PVM. Thus high levels of ROP18 blocked recruitment of IRG to the PVM [Fentress *et al.*, 2010; Fentress and Sibley, 2011]. Other researchers found that ROP18 binds to the C-terminus of the endoplasmic reticu-

lum-bound transcription factor ATF6β triggering its degradation. Normally this transcription factor activates the expression of genes harbouring an unfolded protein response element (UPRE). By inactivating this factor in dendritic cells, *T. gondii* might downregulate the UPRE-mediated host defence. However, the exact target and mechanism remains to be elucidated [Yamamoto *et al.*, 2011].

ROP16, another member of the ROP2 kinase family, was shown to influence the JAK/STAT pathway during an active *T. gondii* infection. Recombinant ROP16 was shown to phosphorylate STAT6. However, only *T. gondii* type I and III, but not type II, can maintain a STAT3/STAT6 activation. A single SNP changing a Leucine to a Serine was shown to change the kinase pocket of ROP16 in type II parasites thus disabling its action. Activation of STAT3/6 leads to reduced IL-12, IL-10 and TNF-α expression by APCs. This in turn leads to a decrease in IFN-γ release and thus reduced killing of the parasite in *T. gondii* type I and II [Melo *et al.*, 2011; Ong *et al.*, 2010].

Another virulence factor was identified as ROP5 which is expressed by all known *T. gondii* isolates, yet the mechanism differs from that of ROP18. ROP5 seems to be expressed as a single protein and comes in three different isoforms, A, B. and C. It is also encoded by a locus of tandemly duplicated genes that are highly polymorphic. Allelic variation of the isoforms was shown to be responsible for a 5-logs difference in virulence in a mouse model [Reese *et al.*, 2011]. Furthermore, type I and type III were shown to be nearly identical and to possess the virulent clusters of ROP5 (5–6 copies), in contrast to type II (9–10 copies). A frameshift in type II ROP5B seemed to result in a non-functional protein. Experiments further revealed that the ROP5 locus is not a single functional unit and that an individual isoform can significantly alter virulence in the absence of the other copies. *T. gondii* type I that had their functional ROP5 removed were shown to be non-virulent. Inserting copies of either isoform of type III ROP5A or ROP5B rescued the previously lost virulence. Bearing in mind that ROP18 was still functional, the authors concluded that ROP5 must act independently of ROP18 or ROP16.

As the ROP5 proteins are present on the cytosolic face of the PVM, they may interact with and thus, deregulate host proteins that are the key to the immune response. However, no target or mode of action has been revealed so far [Behnke *et al.*, 2011; Reese and Boothroyd, 2011; Reese *et al.*, 2011].

It appears, however, that virulence does not necessarily require the inheritance of specific alleles but that reshuffling [Grigg and Sundar, 2009] of existing alleles at only a few loci may be sufficient to produce new generations with different biological traits [Grigg and Boothroyd, 2001; Grigg and Suzuki, 2003]. Virulence may thus be a quantitative trait involving many loci; and the virulence of *T. gondii* could vary depending on the expressed dominant loci and on the specific combination of alleles at the contributing loci which reassort in a crossing event [Grigg and Sundar, 2009].

Importantly, all virulence studies published to date were performed in mouse-models, so no conclusions can be drawn as to the virulence of *T. gondii* isolates in humans or other host species. The present study showed that highly virulent non-canonical *T. gondii* isolates exist or can be formed in Germany. What we do not know is where the oocysts go after being shed by cats and whether the described genotypes can persist in certain host species. This study may be a starting point for more investigations to answer such questions. Are the virulent *T. gondii* clones able to be passed on to other hosts or do they kill wild-type mice or other intermediate host species readily as observed in laboratory mice? If they do so, can they be transmitted to other hosts that are not killed by the parasite, and thus persist in those species?

It seems that a range of different virulence factors determines mouse virulence. So far, the *T. gondii* clones identified in this study were only analysed for UPS-ROP18. Future studies will focus on determining the expression levels of ROP18 and ROP16, as well as direct sequencing of ROP18. Further investigation

of *T. gondii* regarding the activation of STAT3/6 and IRGs phosphorylation will also need to be addressed.

4.3 *T. gondii* in humans from Germany

In contrast to France, screening of pregnant women and new-borns is not compulsory in Germany. Therefore, early detection of human toxoplasmosis is difficult. Furthermore, most infections with *T. gondii* do not cause clinical symptoms and are therefore not detected. All those factors result in very few biopsy samples from patients with toxoplasmosis. Only few samples from patients infected with *T. gondii* were therefore available for this study.

Nevertheless, this is the first study to genotype DNA-samples from patients with ocular toxoplasmosis in Germany. Almost all samples from patients showed alleles of *T. gondii* type II. This reflects the high prevalence of type II in Europe. Since we do not have any information regarding the clinical manifestations in the patients or their age we could not analyse possible associations between genotype and clinical outcome in this group of patients. Previous work suggested that non-canonical or atypical *T. gondii* genotypes were more prevalent in human cases of ocular toxoplasmosis. For the small patient group that was analysed in this study, this hypothesis could not be confirmed. However, the sample size was far too small to draw any final conclusion for Germany.

No data on the onset of symptoms were obtained. It would have been interesting to compare our data on the seasonality of *T. gondii* oocyst shedding by cats to the onset of clinical symptoms in these patients. A study from Serbia revealed that the onset of symptoms such as lymphadenopathy in 39 patients was highest in spring and in October/December, which correlates with our data on *T. gondii* oocyst shedding by cats [Bobic *et al.*, 2010]. Our results also showed that although not all DNA samples could be successfully genotyped, *T. gondii*-DNA was ob-

served in most clinical samples. As already noted in cats, the mere presence of *T. gondii*-DNA as detected by the diagnostic primers of the 529 bp-repeat regions is no guarantee that genotyping can be achieved. Bearing in mind that the diagnostic PCR targets is a repeat region [Homan *et al.*, 2000; Reischl *et al.*, 2003] this seems to be plausible. All genetic regions used for genotyping are based on single copy genes. Thus, detection of these marker regions requires relatively large amounts of DNA. In clinical samples this is not always the case. Genotyping tissue samples of cattle and sheep from Switzerland and of foxes from Germany also highlighted the problem [Berger-Schoch *et al.*, 2011b; Herrmann *et al.*, 2012] of obtaining enough DNA to amplify all genetic marker regions to achieve complete genotyping. From the few loci that were PCR-amplified we cannot confirm that ocular toxoplasmosis is preferentially caused by non-canonical or atypical *T. gondii* in Germany. Several reports suggest that ocular toxoplasmosis is often associated with *T. gondii* of atypical genotypes in the USA [Grigg *et al.*, 2001b]. Considering that type II is also predominately found there, we would have expected similar results for Germany. Genotyping 13 out of 20 patients from France using microsatellite markers showed that most patients with ocular toxoplasmosis were infected with *T. gondii* type II (n = 10). Importantly, three patients showed infections with *T. gondii* types different from type II. One patient showed alleles of types II and III, whereas the *T. gondii* haplotype Africa 1 was identified in two patients. These two patients had a sub-Saharan background or had travelled to China and India a few years previously. This strongly suggested that the patients had contracted the infection with *T. gondii* whilst living in these countries. However, no association of atypical or non-canonical *T. gondii* in patients with ocular toxoplasmosis could be determined [Fekkar *et al.*, 2011]. Reports suggest atypical and other genotypes may be responsible for severe ocular toxoplasmosis in South America, Korea and China [Alves *et al.*, 2010; Quan *et al.*, 2008]. Considering that the overall prevalence of such atypical *T. gondii* genotypes is higher in those countries compared to the European/North American situation, it is not surprising to find patients infected with such *T. gondii* genotypes. However, the finding of

non-canonical *T. gondii* genotypes in British toxoplasmosis patients is remarkable [Aspinall *et al.*, 2003]. Unfortunately, the ethnic background of the patients was not mentioned in the study. They may therefore have observed theses atypical *T. gondii* genotypes in patients with different ethnic background or in patients with extensive travelling history around the world.

An alternative method to circumvent the difficulty of obtaining enough biopsy material for genotyping is serotyping patient sera. Serotyping is based on detecting type-specific differences in short immunodominant regions of proteins (peptides) that are recognised by the immune system. These peptides are 10–20 amino acids long and can be used in ELISA or microarray assays. Antibodies against type-specific *T. gondii* peptides contained in the serum of infected patients bind to such peptides. Depending on the recognised peptides, the serotype of *T. gondii* can be determined [Kong *et al.*, 2003]. However, the technique is not well established and only small numbers of peptides are validated. Such a small number of peptides are not enough to reliably predict the *T. gondii* genotype a patient is infected with. Studies on serotyping can only give a general perspective on the population as a whole. Recent studies comparing the peptide profiles of *T. gondii* infected patients from Europe and those recognized by patients from South America showed that most patients in Europe reacted with peptides specific for *T. gondii* type II. In contrast, patients from Colombia showed positive reactions to peptide specific for type I and type III, confirming some reports on genotyping patients from Europe [Morisset *et al.*, 2008; Nowakowska *et al.*, 2006; Peyron *et al.*, 2006; Sousa *et al.*, 2009]. Results using a newly developed microarray platform for serotyping confirm findings of other groups in Europe and the high prevalence of type II specific peptides recognised by *T. gondii* infected patients from Germany [Maksimov, 2012].

To properly address the question of *T. gondii* genotypes in toxoplasmosis patient in Germany, a concerted effort is needed by all institution diagnosing toxoplasmosis and taking biopsies.

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List of Publications

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Articles in international peer-reviewed journals

First author publications:

Herrmann, D. C.; Wibbelt, G.; Gotz, M.; Conraths, F. J. and Schares, G. (2012): Genetic characterisation of *Toxoplasma gondii* isolates from European beavers (*Castor fiber*) and European wildcats (*Felis silvestris silvestris*), *Veterinary Parasitology*, DOI: 10.1016/j.vetpar.2012.08.026.

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Oral presentations at national and international conferences

- The British Society of Parasitology, Spring Meeting (2012), Glasgow, UK
  Invited speaker: "*Toxoplasma gondii*: genetic diversity around the world and insight into genotypes and virulence of *T. gondii* in Germany"

- Deutsche Gesellschaft für Parasitologie (2012), Heidelberg, Germany
  "*Toxoplasma gondii* genotypes in cats from Germany – genetically different non-canonical *T. gondii* isolated from a single feline sample show high virulences in BALB/c mice"

- The National Symposium on Zoonoses Research (2011), Berlin, Germany
  "Genetically different *T. gondii*, isolated from a single feline faecal sample from Germany, have different virulences in BALB/c mice"

- Deutsche Veterinärmedizinische Gesellschaft-Parasitologie Tagung (2011), Berlin, Germany
  "A single feline faecal sample can contain several genetically different, highly virulent recombinant *Toxoplasma gondii* clones"

- The National Symposium on Zoonoses Research (2010), Berlin, Germany
  "A single mixed *Toxoplasma gondii* oocyst sample from Germany consists of many *T. gondii* clones of different virulences, genetically distinct from the clonal types I, II and III"

- Deutsche Gesellschaft für Parasitologie (2010), Düsseldorf, Germany

"First isolation and characterisation of atypical *Toxoplasma gondii* isolates from cats in Germany"

- The National Symposium on Zoonoses Research (2009), Berlin, Germany
  "*Toxoplasma gondii*: Erster Nachweis, Klonierung und Charakterisierung von atypischen, Typ-II/Typ-III-Rekombinanten in Europa"

- Deutsche Veterinärmedizinische Gesellschaft-Parasitologie Tagung (2009), Leipzig, Germany
  "Molekulare Charakterisierung von *Toxoplasma gondii*–Oozysten in Deutschland"

- British Society of Parasitology, Spring Meeting (2009), Edinburgh, UK
  "Molecular characterisation of *Toxoplasma gondii* in cats in Germany"

- 16th Japanese-German Cooperative Symposium on Protozoan Diseases, (2008), Göttingen, Germany
  "Molecular characterisation of *Toxoplasma gondii* in cats in Germany"

- FEBS International Summer School „Pathogen-Host-Interplay" (2008), Potsdam, Germany
  "Molecular Typing of *Toxoplasma gondii* from cats in Germany"

- 3rd Short Course for Young Parasitologists (2008), Hamburg, Germany
  "Genotyping of *Toxoplasma gondii* in cats in Germany"

Poster presentation at national and international conferences

- Deutsche Gesellschaft für Parasitologie (2012), Heidelberg, Germany
  "*Toxoplasma gondii* prevalence and genotypes in red foxes *(Vulpes vulpes)* and rodents from the German Federal States of Brandenburg and Saxony-Anhalt"

- The National Symposium on Zoonoses Research (2011), Berlin, Germany
  "*Toxoplasma gondii* prevalence and genotypes in red foxes (*Vulpes vulpes*) originating from different regions of Germany"

- Deutsche Veterinärmedizinische Gesellschaft-Parasitologie Tagung (2011), Berlin, Germany
  "Comparison of different commercial DNA extraction kits to detect *Toxoplasma gondii* oocysts in cat faeces"

- Deutsche Gesellschaft für Parasitologie (2010), Düsseldorf, Germany

"Seasonality and spatial distribution of *Toxoplasma gondii*-positive faecal cat samples in Germany"

- The National Symposium on Zoonoses Research (2009), Berlin
  "*Toxoplasma gondii*: Erster Nachweis, Klonierung und Charakterisierung von atypischen, Typ-II/Typ-III-Rekombinanten in Europa"

  "Saisonalität und räumliche Verteilung *Toxoplasma gondii*-positiver Katzenkotproben in Deutschland"

- British Society of Parasitology, Spring Meeting (2009), Edinburgh, UK
  "Molecular characterisation of *Toxoplasma gondii* in cats in Germany"

Appendix

Alignment of DNA sequences of *T. gondii* type I (GT1), type II (ME49), type III (VEG) compared to DNA sequences obtained by sequencing the upstream region of ROP18 of *T. gondii* clones K119/2 2-H8, K119/2 A7, B136/2 C12, K119/2 2-C10, K119/2 G11-C4, B136/5 G12, B136/1 B6-H6; and B136/1 A3-F5. Primer binding sites are underlined; The UPS-ROP18 elements (508 bp element, 44 bp repeat element, UPS, 99 bp element, 109 bp element, ROP18 protein coding region) described by [Khan *et al.*, 2009] are annotated and indicated by a grey background:.

```
                                10        20        30        40
                       ....|....|....|....|....|....|....|....|
Annotations                       ROP18-1F      508 bp element
UPS ROP18 type III     CAGCTAAACTGCCCGCCCTCTTTCATT----CCTCTGTTT
UPS ROP18 type I       ...........................AAGT..CG.....
UPS ROP18 type II      ...........................AAGT..CG.....
K119/2 2-C10           --------------------.......AAGT..CG.....
K119/2 G11-C4          ------------------.........AAGT..CG.....
B136/5_G12             --------...................AAGT..CG.....
B136/1 B6-H6           --------...................AAGT..CG.....
B136/1 A3-F5           ----------------------.....AAGT..CG.....
K119/1 2-H8            ----------------------------------------
K119/2 A7              ----------------------------------------
B136/2 C12             ----------------------------------------

                                50        60        70        80
                       ....|....|....|....|....|....|....|....|
UPS ROP18 type III     CAACGACGTCCATGAGTGCCTTTTTCTTTTCGTTTCCCGA
UPS ROP18 type I       G..........GG..............................
UPS ROP18 type II      G..........GG..............................
K119/2 2-C10           G..........GG..............................
K119/2 G11-C4          G..........GG..............................
B136/5_G12             G..........GG..............................
B136/1 B6-H6           G..........GG..............................
B136/1 A3-F5           G..........GG..............................
K119/1 2-H8            ----------------------------------------
K119/2 A7              ----------------------------------------
B136/2 C12             ----------------------------------------

                                90       100       110       120
                       ....|....|....|....|....|....|....|....|
UPS ROP18 type III     CGCAGCAGCGAACCTAGCTGAATCTTCCAGCTTCGCTTTG
UPS ROP18 type I       ........................A......T.........
UPS ROP18 type II      ..T.....................A......T.........
K119/2 2-C10           ..T.....................A......T.........
K119/2 G11-C4          ..T.....................A......T.........
B136/5_G12             ..T.....................A......T.........
B136/1 B6-H6           ..T.....................A......T.........
B136/1 A3-F5           ..T.....................A......T.........
K119/1 2-H8            ---------..................................
K119/2 A7              ..........................................
B136/2 C12             ----------------------------------------
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                          130       140       150       160
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  AGTTCATAACTCCTTTGTTCTCTCTCTTCCGCCCGTCGGT
UPS ROP18 type I    ..............G.....................C..
UPS ROP18 type II   ..............C.....................A..
K119/2 2-C10        ..............C.....................A..
K119/2 G11-C4       ..............C.....................A..
B136/5_G12          ..............C.....................A..
B136/1 B6-H6        ..............C.....................A..
B136/1 A3-F5        ..............C.....................A..
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          -------------------.....................

                          170       180       190       200
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  TCCCGTCCCTGGTGTCTTCGTGGCATTTTATTCAGTTCTA
UPS ROP18 type I    ....T..................A.........A.......
UPS ROP18 type II   ....T..................A.........A.......
K119/2 2-C10        ....T..................A.........A.......
K119/2 G11-C4       ....T..................A.........A.......
B136/5_G12          ....T..................A.........A.......
B136/1 B6-H6        ....T..................A.........A.......
B136/1 A3-F5        ....T..................A.........A.......
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................

                          210       220       230       240
                    ....|....|....|....|....|....|....|....|
Annotations                           1st 44 bp repeat element
UPS ROP18 type III  TCGCGCCACTCGGACTTTGTTCCTGGCACCCTTTTGTAGC
UPS ROP18 type I    ....................................
UPS ROP18 type II   ....................................
K119/2 2-C10        ....................................
K119/2 G11-C4       ....................................
B136/5_G12          ....................................
B136/1 B6-H6        ....................................
B136/1 A3-F5        ....................................
K119/1 2-H8         ....................................
K119/2 A7           ....................................
B136/2 C12          ......A.............................

                          250       260       270       280
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  TAGGACTCCTTGACGAGTCAGTCAGATTGACGAGGTCGGC
UPS ROP18 type I    ........................C...........
UPS ROP18 type II   ....................................
K119/2 2-C10        ....................................
K119/2 G11-C4       ....................................
B136/5_G12          ....................................
B136/1 B6-H6        ....................................
B136/1 A3-F5        ....................................
K119/1 2-H8         ....................................
K119/2 A7           ....................................
B136/2 C12          ....................................
```

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                                290       300       310       320
                           ....|....|....|....|....|....|....|....|
Annotations                  2nd 44 bp repeat element
UPS ROP18 type III         TA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type I           ..~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II          ..GCTAGGACTCCTTGACGCGTCAGTCAGATTGACGAGGC
K119/2 2-C10               ..GCTAGGACTCCTTGACGCGTCAGTCAGATTGACGAGGC
K119/2 G11-C4              ..GCTAGGACTCCTTGACGCGTCAGTCAGATTGACGAGGC
B136/5_G12                 ..GCTAGGACTCCTTGACGCGTCAGTCAGATTGACGAGGC
B136/1 B6-H6               ..GCTAGGACTCCTTGACGCGTCAGTCAGATTGACGAGGC
B136/1 A3-F5               ..GCTAGGACTCCTTGACGCGTCAGTCAGATTGACGAGGC
K119/1 2-H8                ..~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 A7                  ..~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/2 C12                 ..~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                330       340       350       360
                           ....|....|....|....|....|....|....|....|
Annotations                      3rd 44 bp repeat element
UPS ROP18 type III         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type I           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II          CGGCTAGCTAGGACTCCTTGACGCGTCAGTCAGATTGACG
K119/2 2-C10               CGGCTAGCTAGGACTCCTTGACGCGTCAGTCAGATTGACG
K119/2 G11-C4              CGGCTAGCTAGGACTCCTTGACGCGTCAGTCAGATTGACG
B136/5_G12                 CGGCTAGCTAGGACTCCTTGACGCGTCAGTCAGATTGACG
B136/1 B6-H6               CGGCTAGCTAGGACTCCTTGACGCGTCAGTCAGATTGACG
B136/1 A3-F5               CGGCTAGCTAGGACTCCTTGACGCGTCAGTCAGATTGACG
K119/1 2-H8                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 A7                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/2 C12                 ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                370       380       390       400
                           ....|....|....|....|....|....|....|....|
Annotations                      UPS-ROP18
UPS ROP18 type III         ~~~~~~~~~~TTTACGAGAGACTGAAGCGTTGCCAGAAGC
UPS ROP18 type I           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II          AGGCCGGCTA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10               AGGCCGGCTA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4              AGGCCGGCTA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12                 AGGCCGGCTA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6               AGGCCGGCTA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5               AGGCCGGCTA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8                ~~~~~~~~~~.............................
K119/2 A7                  ~~~~~~~~~~.............................
B136/2 C12                 ~~~~~~~~~~.............................
```

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                          410       420       430       440
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  GGGGAATTTTTGTTTCTTCCTTTCTTCCCCAGAAAATTCG
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          450       460       470       480
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  AGAAGCGACTTGAGGTCGGACAAGGCAGTGGGCTGGGCAT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          490       500       510       520
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  GCAGTGAAAAGCACTGTTTTCGTCTGAAACCGTTGCTCGG
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................

                          530       540       550       560
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  GATGTTGCTCAATGGAGTCATGGCACCGCATTCCTTTTTG
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
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                          570       580       590       600
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   AAATGTGCCTCATGCCTTCTGTCCGTGCGTATTTTGTCCC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................

                          610       620       630       640
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   TGTACCTGAATACTTACGGGATGCACTGGTCAGTTTCAGG
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................

                          650       660       670       680
                     ....|....|....|....|....|....|....|....|
Annotations                                    ROP18-2F
UPS ROP18 type III   CAGGATGATTGGTGCCGCCAACGTGCATCTTCGGGTAACA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ..................................T...
K119/2 A7            ........................................
B136/2 C12           ........................................

                          690       700       710       720
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   AACGTGAAGCACGTGTCACTAAGAAGAGGTTCCACTGACC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
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                          730       740       750       760
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   CTTTATATCCTGTGGCCATCCCCTATGCATCAGTTATTCA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          770       780       790       800
                     ....|....|....|....|....|....|....|....|
Annotations                          ROP18-1R
UPS ROP18 type III   GGGTGTTTTGTCAGTCAGGTTAGCCGGAAGTTTCCATCTA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          810       820       830       840
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   AACAGACACACTAAAGCGCCTAGGGTACTGAGTATGACTC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          850       860       870       880
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   AAGAAGCACTTTTCTATCGTAGGTTCGGTCTCACTAGGAA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
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                          890       900       910       920
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   TTAGAACAGCATGTCAAGTTCTTTCCCTGTTGTTACGCAT
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................

                          930       940       950       960
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   TATCGATGTACGCTTTTTACGCACGCTGTGCACTCATGTA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................

                          970       980       990       1000
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   CAGCAACCACAAAGGAGTTGATTGTCGACGAAGACGTTAG
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................

                          1010      1020      1030      1040
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   TTCAGAGAAATTGAACGAAAGAAAGTCACATAGAAACGTT
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................
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                          1050      1060      1070      1080
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  TTTCGCTCAACGCAGCCAGCACGCTAGTGTCCTTCGGGGT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          1090      1100      1110      1120
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  AGTCAGTAAACACAAAACTGACTGGTGTCAAACCGATACA
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          1130      1140      1150      1160
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  CATGATGCCATTTCTTACGGCTGGTCCAGAGTCTGCATTC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          1170      1180      1190      1200
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  ACCTACGGATGAGTACAGTGGTACGAACAGTCAGGGAAAA
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
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                          1210      1220      1230      1240
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  AATTGAACGGGTAGTTTCAGTAAACCTGTGTTCGTAGTTT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................

                          1250      1260      1270      1280
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  CTTCACACACTGGAGACAACCTTCCTGACACGTCGCTCCT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................

                          1290      1300      1310      1320
                    ....|....|....|....|....|....|....|....|
Annotations                     ROP18-3F
UPS ROP18 type III  GCTTGTACGCGGTTGTAGCTCCTCTGAATTCAGGATTTTG
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................

                          1330      1340      1350      1360
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  TGGTAGTGTCACGCTATACCGCTTCTCGGCTCTAGCTGTC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
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                          1370      1380      1390      1400
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   GAGTATCCTGGTGCTTCTTACTTGGCAGGCGAGACAGTGA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          1410      1420      1430      1440
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   CCGCTCCTGGTATACCCACGGTGCCACTGCGCAAACAGAA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ......................CCC...............
                          1450      1460      1470      1480
                     ....|....|....|....|....|....|....|....|
Annotations                                      ROP18-2R
UPS ROP18 type III   TGGAATTGTAGAAATACTGAACAACTTGAGAAGATGGCGA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          1490      1500      1510      1520
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   GCGCCCGGCTGCCTGTGATAGACGGTTTCAGTGAACAAAA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
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                          1530      1540      1550      1560
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  ATAAGAACTCACTTAACTGCAACAGCCATTGTTGGTATAT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................

                          1570      1580      1590      1600
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  CTGTGCATCAAGAATATTCGACCGGGCAACCATCCTGTAT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................

                          1610      1620      1630      1640
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  TGCGAATAGGCAAACGACCATTTAACGTTCAAGTCGTTGC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................

                          1650      1660      1670      1680
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  CTGCCTTCAGTCCACATGTATGGTGTAGAGACCGTCTTTC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................
```

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                          1690      1700      1710      1720
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   ACTTGATGGTTGCTTGCTACAGCGCACTTTCGTGTTCGAC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          1730      1740      1750      1760
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   GCCATAGGCGGTACATCCATTACTCGTAAGGAAAGGGAAC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           .......-................................
                          1770      1780      1790      1800
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   TATCTATCACAGGCAGTTTGGTGAATTGCGGTCGGTCACG
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
                          1810      1820      1830      1840
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   CACAGATCCATTCCACAACCTAACTCTGGAGGTAAACGCT
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
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                          1850      1860      1870      1880
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   GACTACTCCACGTGTGGCACAAACAAGGTGTCTGGCCTTT
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................

                          1890      1900      1910      1920
                     ....|....|....|....|....|....|....|....|
Annotations                                    ROP18-4F
UPS ROP18 type III   GTTTGCAAATAGACAGAGATGTTACCGTCCTCCGTTTTTC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................

                          1930      1940      1950      1960
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   TCTTTTTTCGTGGCTCATCTAAATGCGAAGTGCCGGTGAA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................

                          1970      1980      1990      2000
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   GTGTGTGCAACGAGTAGGGCACGCAAATATACAGCTTCAA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ........................................
K119/2 A7            ........................................
B136/2 C12           ........................................
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                          2010      2020      2030      2040
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  AATGCCTGCCACTACGCAGTTCCTGAAAACAGTGCGTCGC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          2050      2060      2070      2080
                    ....|....|....|....|....|....|....|....|
Annotations                                     ROP18-3R
UPS ROP18 type III  TTTCGGAACCCAAGACTGGCTTCAAAGATGCAGCCAACGA
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          2090      2100      2110      2120
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  GACAAGCGTGACCGCGCAGCTGGCCATCTGTACGTCTTCA
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
                          2130      2140      2150      2160
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  CTTCCTCTATCTATTTCTGTTGGAGATGCTCTTGAGATGC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ........................................
K119/2 A7           ........................................
B136/2 C12          ........................................
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                          2170      2180      2190      2200
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   CTCCCGTTCTCCACTCAACTTTGCTGTCTGGTTGAGCACC
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................

                          2210      2220      2230      2240
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   ACAGTTCACTGTGAGACGCTTCACGAAAAGTGTTCCCCTA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................

                          2250      2260      2270      2280
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   GTCGACGCTTGGCACCGTTCGAGATAAATTAAACTGTCGG
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................

                          2290      2300      2310      2320
                     ....|....|....|....|....|....|....|....|
UPS ROP18 type III   CATTTGCGAATATGGCAAGGAATATGGAAAATAGTTTTTA
UPS ROP18 type I     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8          ...................................
K119/2 A7            ...................................
B136/2 C12           ...................................
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                          2330      2340      2350      2360
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  ACACGGATACATAGGAAGACATACAGTTAATCTTTTTATC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................

                          2370      2380      2390      2400
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  GACATCCCGCTTCTTCTAGCAGACGGAGAGTCAGGCCTTT
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................

                          2410      2420      2430      2440
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  AAGCTGTTGTTCACAAATTTCGAGCGCCAGTCACCAGAAC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................

                          2450      2460      2470      2480
                    ....|....|....|....|....|....|....|....|
UPS ROP18 type III  GACCCGGTTTCCAGTGGCACCAGCAAGGCCTCCCTAAATC
UPS ROP18 type I    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8         ...................................
K119/2 A7           ...................................
B136/2 C12          ...................................
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                          2490      2500      2510      2520
                         ....|....|....|....|....|....|....|....|
UPS ROP18 type III       CGGAAGCAACTATAAGTAGTCGTAAGCAGCTCGAAGGGCA
UPS ROP18 type I         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8              ........................................
K119/2 A7                ........................................
B136/2 C12               ........................................
                          2530      2540      2550      2560
                         ....|....|....|....|....|....|....|....|
UPS ROP18 type III       ACACAGCATGAGCTTAAGAGTTGAAAAAGTCAGTGAAGTT
UPS ROP18 type I         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type II        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 2-C10             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 G11-C4            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/5_G12               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 B6-H6             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/1 A3-F5             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/1 2-H8              ........................................
K119/2 A7                ........................................
B136/2 C12               ........................................
                          2570      2580      2590      2600
                         ....|....|....|....|....|....|....|....|
                                                  99 bp element
UPS ROP18 type III       CTTTTGGTAGAGAAAGCCATGAAGCGCTGAAGG~~~~~~~
UPS ROP18 type I         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
UPS ROP18 type II        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
K119/2 2-C10             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
K119/2 G11-C4            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
B136/5_G12               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
B136/1 B6-H6             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
B136/1 A3-F5             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GCCACGC
K119/1 2-H8              .................................~~~~~~~
K119/2 A7                .................................~~~~~~~
B136/2 C12               .................................~~~~~~~
                          2610      2620      2630      2640
                         ....|....|....|....|....|....|....|....|
UPS ROP18 type III       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type I         TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
UPS ROP18 type II        TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
K119/2 2-C10             TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
K119/2 G11-C4            TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
B136/5_G12               TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
B136/1 B6-H6             TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
B136/1 A3-F5             TATGCACCTCTTGCATACAATTGTTGTAGACAGCATGGTG
K119/1 2-H8              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 A7                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/2 C12               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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                                2650      2660      2670      2680
                         ....|....|....|....|....|....|....|....|
UPS ROP18 type III       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
UPS ROP18 type I         GTGTGCGAGACACTGCAGTCAAATGGCTCACCCAACGCCG
UPS ROP18 type II        GTGTGCGAGACACTGCAGTCAAATGCCTCACCCAACGCCG
K119/2 2-C10             GTGTGCGAGACACTGCAGTCAAATGCCTCACCCAACGCCG
K119/2 G11-C4            GTGTGCGAGACACTGCAGTCAAATGCCTCACCCAACGCCG
B136/5_G12               GTGTGCGAGACACTGCAGTCAAATGCCTCACCCAACGCCG
B136/1 B6-H6             GTGTGCGAGACACTGCAGTCAAATGCCTCACCCAACGCCG
B136/1 A3-F5             GTGTGCGAGACACTGCAGTCAAATGCCTCACCCAACGCCG
K119/1 2-H8              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
K119/2 A7                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
B136/2 C12               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                2690      2700      2710      2720
                         ....|....|....|....|....|....|....|....|
                                        108 bp element
UPS ROP18 type III       ~~~~~~~~~~~~~TCGGCTACTCACGCTATGCTCGGCAGA
UPS ROP18 type I         CGTCTCATTCTTCCAA.AAC..GT.CG.G.CT...A....
UPS ROP18 type II        CGTCTCATTCTTCCAAAAAC..GT.CG.G.CT...A....
K119/2 2-C10             CGTCTCATTCTTCCAAAAAC..GT.CG.G.CT...A....
K119/2 G11-C4            CGTCTCATTCTTCCAAAAAC..GT.CG.G.CT...A....
B136/5_G12               CGTCTCATTCTTCCAAAAAC..GT.CG.G.CT...A....
B136/1 B6-H6             CGTCTCATTCTTCCAAAAAC..GT.CG.G.CT...A....
B136/1 A3-F5             CGTCTCATTCTTCCAAAAAC..GT.CG.G.CT...A....
K119/1 2-H8              ~~~~~~~~~~~~~.....................
K119/2 A7                ~~~~~~~~~~~~~.....................
B136/2 C12               ~~~~~~~~~~~~~.....................

                                2730      2740      2750      2760
                         ....|....|....|....|....|....|....|....|
UPS ROP18 type III       TTGATACAGCCGTTGACAAAGCACTACCATATTTTACAGT
UPS ROP18 type I         ......................A.................
UPS ROP18 type II        ......................A.................
K119/2 2-C10             ......................A.................
K119/2 G11-C4            ......................A.................
B136/5_G12               ......................A.................
B136/1 B6-H6             ......................A.................
B136/1 A3-F5             ......................A.................
K119/1 2-H8              ........................................
K119/2 A7                ........................................
B136/2 C12               ........................................

                                2770      2780      2790      2800
                         ....|....|....|....|....|....|....|....|
UPS ROP18 type III       TTTGTACTCACCCGAGTACAGTTTGTGTGAATGTTGTAAT
UPS ROP18 type I         ............C...C............A.....G..
UPS ROP18 type II        ............C...C............A.....G..
K119/2 2-C10             ............C...C............A.....G..
K119/2 G11-C4            ............C...C............A.....G..
B136/5_G12               ............C...C............A.....G..
B136/1 B6-H6             ............C...C............A.....G..
B136/1 A3-F5             ............C...C............A.....G..
K119/1 2-H8              ........................................
K119/2 A7                ........................................
B136/2 C12               ........................................
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                                2810      2820      2830      2840
                       ....|....|....|....|....|....|....|....|
                           ROP18 trascriptional start
UPS ROP18 type III     GTTTTCGGTACAGCGGCCATCTCTTACGTGTACCGTCGTC
UPS ROP18 type I       ...................C........C...........
UPS ROP18 type II      ...................C........C...........
K119/2 2-C10           ...................C........C...........
K119/2 G11-C4          ...................C........C...........
B136/5_G12             ...................C........C...........
B136/1 B6-H6           ...................C........C...........
B136/1 A3-F5           ...................C........C...........
K119/1 2-H8            .......................----------------
K119/2 A7              ........................................
B136/2 C12             ..--------------------------------------

                                2850      2860      2870      2880
                       ....|....|....|....|....|....|....|....|
                                                      ROP18-4R
UPS ROP18 type III     CGAATGGGTTTAGCGACTCTTCTCCGGAAGACAGCCTGTC
UPS ROP18 type I       ........................C...............
UPS ROP18 type II      ........................C...............
K119/2 2-C10           ........................C.........-----
K119/2 G11-C4          ........................C...............
B136/5_G12             ........................C...............
B136/1 B6-H6           ........................C...............
B136/1 A3-F5           ........................C...............
K119/2 A7              ...........-----------------------------

                                2890
                       ....|....|....|....
UPS ROP18 type III     TTGCGGGGTTAACTGTAGC
UPS ROP18 type I       ............A......
UPS ROP18 type II      ......T.....A......
K119/2 G11-C4          ......-------------
B136/5_G12             ..-----------------
B136/1 B6-H6           .------------------
B136/1 A3-F5           .....--------------
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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

Berlin, den 26.04.2012

Daland C. Herrmann